

# "LEGIONNAIRES"

the disease, the bacterium  
and methodology



U.S. DEPARTMENT OF  
HEALTH, EDUCATION, AND WELFARE  
PUBLIC HEALTH SERVICE  
CENTER FOR DISEASE CONTROL  
ATLANTA, GEORGIA 30333



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# "LEGIONNAIRES"

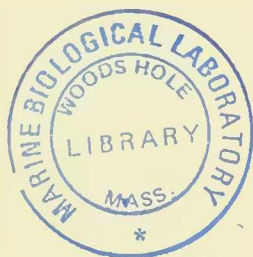
## the disease, the bacterium and methodology

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# Foreword



**L**egionnaires' disease and its causative agent are subjects of worldwide concern. This is evident from the response to an illness whose cause could not initially be defined and from the efforts of the clinical, medical, and general health surveillance communities around the world to treat patients with Legionnaires' disease effectively in order to minimize the threat posed to the public health.

The staff at the Center for Disease Control (CDC) responded quickly and continues to respond to the need for more complete information on Legionnaires' disease, the bacterium which causes it, methods for confirming a presumptive diagnosis, and the most appropriate therapy.

The Legionnaires' disease bacterium is special—it does not follow the usual rules. It is a bacterium but remains invisible with traditional bacteriological techniques; it does not grow on the usual bacterial media, and it does not stain with the usual bacterial stains. It grows both inside and outside cells; it mimics some of the laboratory characteristics of rickettsiae, and the clinical characteristics of viruses. This organism does not even seem to follow the usual rules in causing illness; it causes pneumonia under some conditions but not under others, and when it does cause pneumonia, it may mimic viral pneumonia but at the same time leave a few small tantalizing bacterial clues such as an increase in the white blood count.

We had not identified an important new human bacterial pathogen since the 1950's, but now we must ask how many others there might be that do not follow the rules—a real challenge to all microbiologists and clinicians to reevaluate techniques.

This manual is an effort to disseminate knowledge gained at CDC in the hope that it will be used by the health community for rapid identification of the bacterium and prompt treatment as well as prevention of the illness. It is also hoped that others in academic and research settings will build on the information presented here to further improve diagnostic tools.

A handwritten signature in cursive script that reads "William H. Foege". The signature is written in dark ink and is positioned above the printed name and title.

William H. Foege, M.D.  
Director, Center for Disease Control

# Acknowledgements

We are indebted to Dr. John H. Krickel, Director, Laboratory Training and Consultation Division, Bureau of Laboratories, whose guidance, support, and encouragement made this manual a reality.

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We are further indebted to Dr. Roslyn Q. Robinson, Director, Bureau of Laboratories, Dr. Philip S. Brachman, Director, Bureau of Epidemiology, and many staff members from both Bureaus whose cooperation and support were vital to the development of this manual.

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# Preface

This laboratory manual was designed to be used in training activities related to Legionnaires' disease methodology and as such is intended to be a working model. It represents a concerted effort to compile useful information for the medical community on the progress which has been made at the Center for Disease Control (CDC) in defining a disease entity and its causative agent.

The history and nature of Legionnaires' disease are discussed on the basis of evidence gathered through examination of the clinical and pathological features of the disease.

All of the known characteristics of the Legionnaires' disease bacterium (LDB), including cellular fatty acid composition, antimicrobial susceptibility, and deoxyribonucleic acid (DNA) hybridization, are described in a series of chapters. Brenner et al. proposed the classification *Legionella pneumophila* sp. nov. for the LDB after evaluating the results of their extensive DNA relatedness studies with LDB strains from serogroups 1, 2, and 3. The recently described serogroup 4, as well as some future LDB isolates, may or may not be closely related to these first three serogroups; therefore, until proven to be *L. pneumophila*, they should be designated by the broader term LDB. Since a clear-cut distinction is not always made between serogroups 1-3 (*L. pneumophila*) and serogroup 4, the editors and authors have used the more general and familiar designation LDB when discussing the causative organism of Legionnaires' disease.

Specific procedures are detailed for animal and egg inoculation, media preparation and use, direct and indirect immunofluorescence, and various other methods for safely testing clinical and environmental material. Although modifications in technique will continue to appear as research progresses, the procedures outlined in this manual have proven reliable and can be used diagnostically.

Another section of the manual catalogs most aspects of the CDC assistance program pertaining to Legionnaires' disease. Included are policies regarding technical support and consultation and procedures to follow in obtaining materials and services beyond the scope of many institutions.

The final section of the manual contains a reading bibliography as a general reference. We realize that the rapidity with which new material on Legionnaires' disease is being published makes any such listing incomplete, but it is offered to the reader as a reference base.

Although the material presented in this manual reflects the "state of the science" relative to the LDB at CDC, the editors wish to stress the fact that this is not an all-inclusive publication. Valuable work is being reported from other institutions, and we urge the reader to utilize these resources as they become available.

In addition to the contributors listed elsewhere, this manual could not have been produced without the assistance of Glenda S. Cowart and Thena M. Durham of the Biological Products Division, Bureau of Laboratories; the staff of Publications Management Branch, and Louise Lewis, CDC Library, Office of the Center Director; Fay Neal, Gerri Stedman, and Lois Jennings of the Laboratory Training and Consultation Division, and Claudia Lewis, Effie Spencer, Donna Mills, and Barbara Gary of the Word Processing Activity, Bureau of Laboratories.

Gilda L. Jones  
G. Ann Hébert  
Editors



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# **PART ONE**

## **The Disease**



# **Clinical Manifestations of Legionnaires' Disease and Recommended Therapy**

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# Clinical Manifestations of Legionnaires' Disease and Recommended Therapy

*William B. Baine*

Legionnaires' disease (LD) has most commonly been recognized as a form of pneumonia. Symptoms of this syndrome usually become apparent 2 to 10 days after known or presumed exposure to airborne Legionnaires' disease bacteria (LDB). The earliest symptoms are malaise, myalgia, and mild headache. A non productive cough is common, but sputum production is sometimes associated with the disease. Within less than a day the patient may experience rapidly rising fever and the onset of chills. Although physical examination of the patient may reveal rales on auscultation, fever to 39°- 41°C (102°-105°F), and relative bradycardia, no physical findings are specific to this disease. Associated manifestations may include confusion, chest pain, abdominal pain, impaired renal function, and diarrhea.

Routine laboratory tests commonly reveal a moderate leukocytosis with a shift to the left in the granulocyte series. Proteinuria, hyponatremia, hypophosphatemia, azotemia, elevated aminotransferase levels, and a high erythrocyte sedimentation rate are often seen in various combinations. Radiographs of the chest reveal patchy infiltrates that may progress to extensive consolidation. Cavitory pneumonia is uncharacteristic, but some examples have been reported. A case-fatality rate of approximately 15% for sporadic cases has been reported in the United States.

Two LD case histories are presented below:

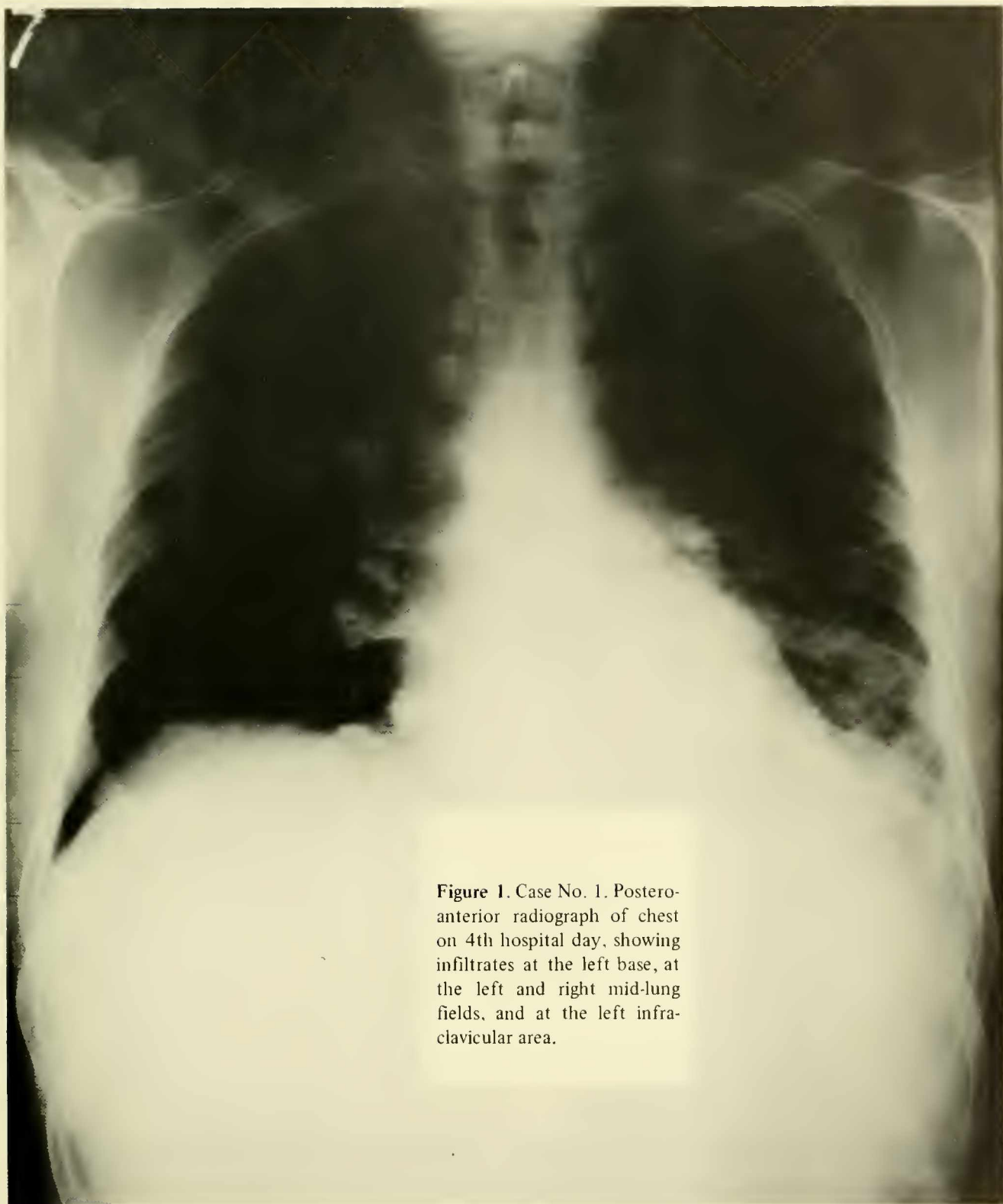
## **Case History #1.**

A 59-year-old construction worker was hospitalized July 5 in Missouri with a 5-day history of malaise, myalgia, chills, and fever to 105°F after a 2½ week vacation trip to Canada, the western United States, and Mexico. On this trip he had gone boating; visited a sawmill, a zoo, and the engine room of a ship; fed birds in a public park; and repaired some malfunctioning air conditioners in a hotel. He did not smoke, and his past medical history was unremarkable except for two episodes of pneumonia in childhood and a case of malaria while serving in the South Pacific during World War II.

The physical examination was unremarkable except for the presence of fever and of occasional rales in the left lung.

Admission urinalysis revealed a specific gravity of 1.033, pH 6, 1+ protein, 6-8 white cells, and 3-4 red cells. The admission hematocrit was 44% with a hemoglobin of 14.2 g/dl, and 13,300 white cells/nl. The white cell differential was 72% neutrophils (including 3% bands), 4% monocytes, and 24% lymphocytes with an adequate number of platelets. Blood chemistries were generally within normal limits. The chest radiograph on admission revealed an infiltrate at the left base.

He was treated with cephalixin, penicillin, and glucocorticoids and continued to have hectic fevers. Within 3 days the infiltrate at the left base had partially resolved, but new infiltrates were present in the left mid-lung field, the left infraclavicular area, and the right mid-lung field (Figure 1). A lung biopsy performed on the 11th hospital day revealed consolidation and a severe inflammatory reaction with a significant polymorphonuclear component. After 8 days in the hospital, the patient became afebrile. Routine attempts to



**Figure 1.** Case No. 1. Postero-anterior radiograph of chest on 4th hospital day, showing infiltrates at the left base, at the left and right mid-lung fields, and at the left infra-clavicular area.





define a microbial etiology were unsuccessful. The patient's condition improved greatly, and he was discharged on the 15th hospital day with a diagnosis of pneumonia of undetermined etiology, possibly caused by a virus or the LDB. From July 8 to July 18 his antibody titer to the LDB (serogroup 1) by indirect immunofluorescence rose from 32 to 256.

### Case History #2.

A 52-year-old British tourist experienced malaise and fever on September 15 while vacationing in Spain. He returned to England on September 20. Three days later he developed cough, shortness of breath, and diarrhea. On admission to a hospital on September 24, he was confused and had a temperature of 40°C and signs of right-sided pneumonia. His chest radiograph showed infiltrates out of proportion to the clinical findings, and his leukocyte count was normal; he was therefore treated for primary atypical pneumonia with erythromycin. His fever lysed within 3 days, but he remained confused, and a lumbar puncture and an encephalogram were done because encephalitis was suspected. No abnormalities were found, and by October 7 the patient was asymptomatic except for residual asthenia. Routine microbiological diagnostic tests were unrevealing. Indirect immunofluorescence was used to document the patient's seroconversion to the LDB (serogroup 1) antigen.

Legionnaires' disease may also take the form of a self-limited illness with fever, myalgia, malaise, and headache, but with few or no respiratory manifestations and no pneumonia. This syndrome, originally termed "Pontiac fever," is remarkable for the absence of associated fatalities. The reason for the difference between the pneumonic and Pontiac fever syndromes of LD remains conjectural.

Symptomatic therapy suffices for patients with the Pontiac fever syndrome. Specific treatment for pneumonia caused by the LDB is not based on controlled clinical trials of human illness, but available evidence suggests the erythromycin reduces the risk of fatality among patients with pulmonic LD. Doses of up to 1 gram every 6 h of erythromycin gluceptate or lactobionate can be given intravenously as the initial therapy for seriously ill patients. Note that the intravenous formulations of erythromycin must be reconstituted in Sterile Water for Injection without bacteriostatic preservatives. The dose can be lowered, and the medication can be given orally as the patient's condition stabilizes. The optimal duration of treatment remains unclear; some clinicians experienced in treating patients with LD continue antibiotic therapy for 3 wk or longer, but this is not a universal practice. The dosage of erythromycin must be adjusted for patients with severe hepatic disease or renal failure. An alternative to erythromycin is a tetracycline. Rifampin is also effective against the LDB in several laboratory models, but it should only be prescribed for LD patients as a supplement to erythromycin if the patients have not responded well to a single antibiotic. It could also be used in conducting properly organized trials to compare erythromycin therapy with erythromycin-rifampin therapy. Treatment with penicillins, cephalosporins, or aminoglycosides does not appear to benefit patients with LD.

Supportive therapy is as important as antibiotic treatment for patients with LD. Those with pneumonia should have their arterial blood gases monitored, and oxygen therapy and assisted ventilation should be provided as indicated. Attention to fluid and electrolyte balance is mandatory—particularly for patients with high fever, diarrhea, or disturbances of consciousness. Patients who have hypotension or shock may need to have carefully monitored urine output, central venous pressure, or pulmonary capillary wedge pressure, and intra-arterial blood pressure to provide a guide for intravenous fluid administration and vasopressor therapy. Renal function should be followed carefully during the acute illness; an occasional patient requires dialysis until renal function is restored. Stuporous and comatose patients require special nursing care to prevent conjunctival desiccation, parotitis, aspiration of gastric contents, urinary retention, fecal impaction, and pressure sores.

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# Pathologic Features of Legionnaires' Disease

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## Pathologic Features of Legionnaires' Disease

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Both autopsy and biopsy specimens from patients with Legionnaires' disease (LD) have been examined in the Pathology Division laboratory at the Center for Disease Control. The only consistent pathologic findings in autopsies of patients dying with acute LD were in the lungs where a pneumonia was present. This pneumonia was frequently confluent, often involving one or more lobes in their entirety. In lung specimens the diseased tissue has appeared gray and granular. Fibrinous pleuritis has frequently been present.

Microscopically, the histologic pattern of tissues examined at autopsy from patients with LD has been principally that of an acute fibrinopurulent pneumonia which resembles the hepatization stages of lobar pneumonia. Of particular note is the exudation of neutrophils, macrophages, and large amounts of fibrin into the alveolar spaces (Figs. 1 and 2). Often this material can be seen passing through pores of Kohn. In some cases there is extensive necrosis of the inflammatory exudate. The extent of inflammatory cell necrosis varies, and the observer should not be surprised to find areas and sometimes entire lungs in which the cells are quite intact. The underlying lung structure remains relatively undisturbed in acute pneumonias caused by the Legionnaires' disease bacterium (LDB). However, focal interstitial necrosis, which is virtually nonexistent in uncomplicated pneumococcal lobar pneumonia, is occasionally seen in LD. The degree to which red blood cells extravasate into the alveoli varies. Most sections contain few or no intra-alveolar erythrocytes, but areas of moderate to severe hemorrhage are occasionally present. In LD pneumonia, the inflammatory process has not been noted to involve blood vessel walls or large bronchi. Interstitial infiltration during the acute stage of the disease is minimal.

The staining qualities of the LDB are unusual. It does not stain with hematoxylin and eosin and stains only rarely with standard tissue stains for bacteria, such as the Brown-Brenn, Brown-Hopps, and MacCallum-Goodpasture procedures. However, the organism can be demonstrated consistently with the Dieterle silver impregnation procedure (Fig. 3), a fact which makes it the staining method of choice for the LDB in our laboratory. Other investigators have reported to us that the Warthin-Starry and other silver impregnation techniques are also effective. Some institutions have used various modifications of the Giemsa stain with some success in demonstrating the LDB in paraffin sections, but in our hands this procedure has not been satisfactory. Even when the organisms can be stained with this method, it is difficult to obtain adequate color differentiation between the bacterium and the inflammatory background. The Giménez stain is useful for impression smears and frozen sections but is unsatisfactory for paraffin-embedded tissue.

In tissue sections stained by the Dieterle silver impregnation procedure the LDB's appear as short, pleomorphic rods (Fig. 4) measuring 2 to 4 microns in length and up to 1 micron in diameter. Some of these rods appear bipolar, and beaded forms are noted. Organisms are diffusely distributed throughout the areas affected by the acute pneumonia, but concentrations vary from patient to patient. The degree of necrosis of the inflammatory exudate is directly related to the number of organisms that can be demonstrated. Clusters of organisms are commonly observed within macrophages in Dieterle-stained sections. In some cases, by focusing up and



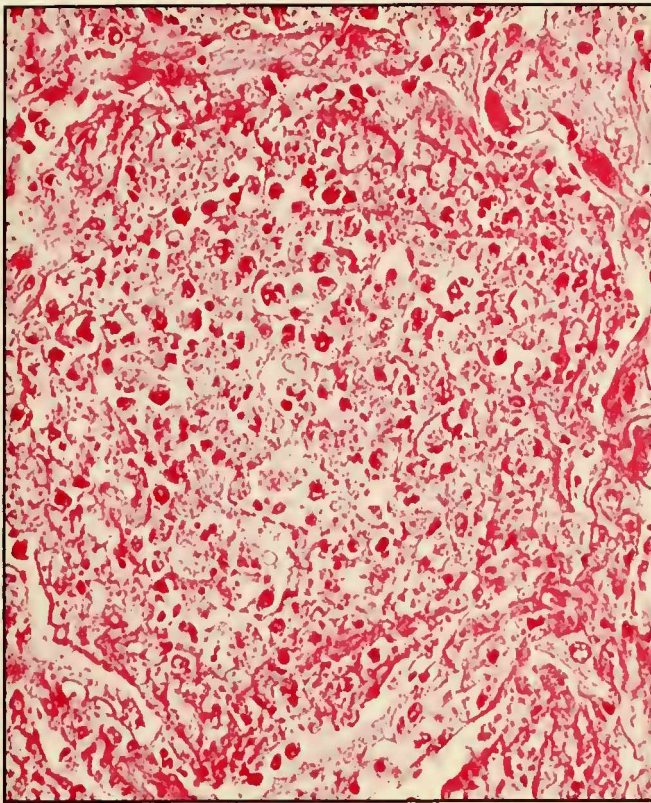


Figure 1. Intra-alveolar exudate filling alveolar space. Note necrosis of inflammatory cells (hematoxylin-eosin, original magnification X 100).

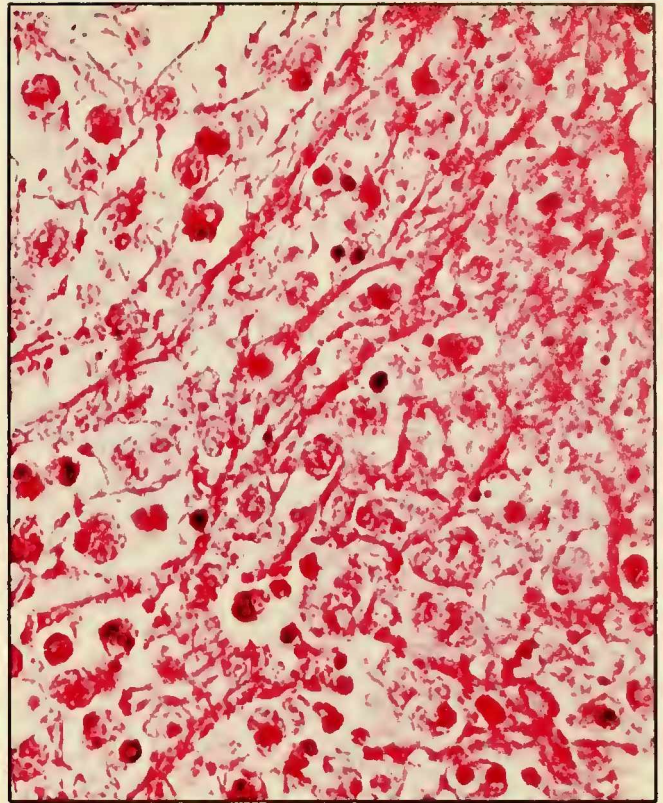


Figure 2. Detail of inflammatory exudate. Note mixture of polymorphonuclear leukocytes, macrophages, and fibrin (hematoxylin-eosin, original magnification X 200).

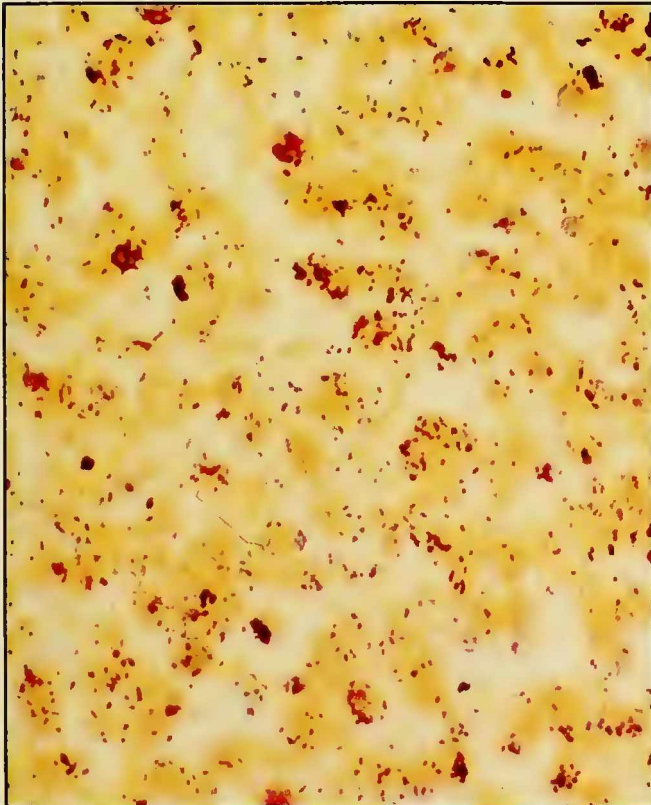


Figure 3. Legionnaires' disease bacteria in area of consolidation. (Dieterle silver impregnation, original magnification X 200).

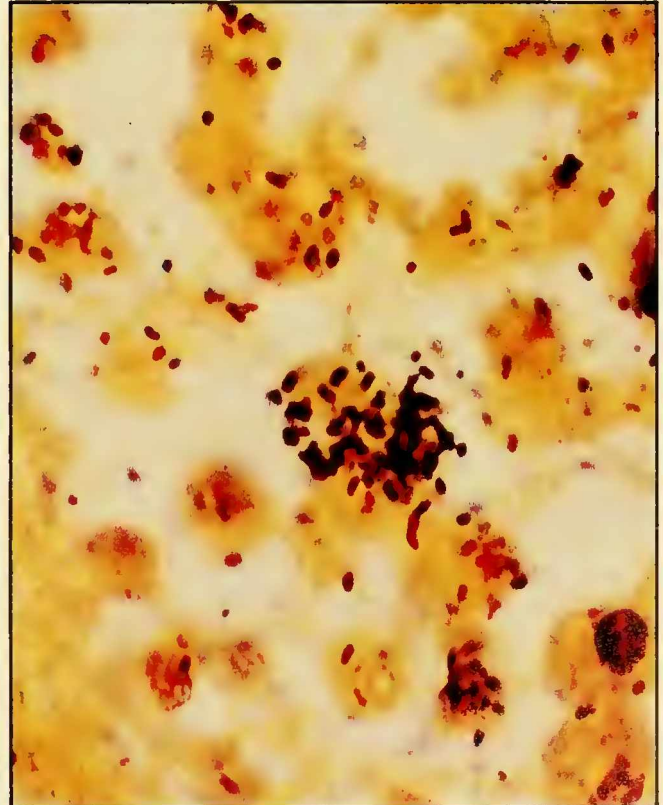


Figure 4. Legionnaires' disease bacteria. Note that they appear as short, blunt rods (Dieterle silver impregnation, original magnification X 500).



down through individual cells under oil immersion or by examining tissue with the electron microscope, 50 or more organisms can be observed in individual macrophages. Many extra-cellular forms can also be seen.

We have examined a number of surgical specimens from patients who had serologically confirmed LD. In some, the histologic pattern was identical to that of the acute fibrinopurulent pneumonia previously described for autopsy specimens. In other cases an organizing pneumonia with varying degrees of interstitial fibrosis has been present. We have been unsuccessful in our efforts to demonstrate organisms in these organizing pneumonias using silver impregnation or immunofluorescent staining, and in these cases the diagnosis of LD has been confirmed retrospectively by seroconversion. Organization may occur late in the course of the disease in an unknown percentage of patients and may result in serious pulmonary functional impairment.

The pathologic features described here cannot serve as the sole basis for diagnosing LD. However, such histologic patterns in lung tissue certainly indicate the need to rule out or confirm a diagnosis of this disease. A definitive diagnosis of LD must be obtained on the basis of results from the more specific laboratory methods described in other sections of this manual.

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# The Epidemiology of Legionnaires' Disease

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# The Epidemiology of Legionnaires' Disease

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The term "Legionnaires' disease" (LD) is currently used to refer to illness caused by the bacterium that caused the 1976 outbreak of respiratory illness in Philadelphia or by another strain of the same organism. Legionnaires' disease can occur in large common-source outbreaks, as a series of cases in areas of apparently high endemicity, or as sporadic cases without apparent temporal or geographic clustering.

The earliest documented outbreak of LD occurred in 1965 at St. Elizabeth's Hospital, a large psychiatric facility in Washington, D.C. Eighty-one patients became ill, and 14 died. Epidemiologic evidence suggested a link between infection and wind-blown dust from excavations on the hospital grounds. In 1968 at least 144 cases of self-limited illness occurred in employees or visitors who had entered a health department building in Pontiac, Michigan. Investigation at that time demonstrated that the etiologic agent was present in water in the evaporative condenser of a malfunctioning air-conditioning system. The Pontiac agent is now known to have been the Legionnaires' disease bacterium (LDB). In July 1973, an estimated eight Scottish vacationers contracted LD while vacationing in Benidorm, Spain; three died. In 1974 at least 20 persons attending an Oddfellows Convention at the Bellevue Stratford Hotel in Philadelphia developed pneumonia, and two died. This outbreak has since been shown to have been a cluster of cases of LD.

The existence of the LDB was first recognized in laboratory investigations of a large outbreak of pneumonia among persons associated with Philadelphia's Bellevue Stratford Hotel in 1976. Of those who were known to have been inside the hotel at some point during an American Legion Convention, 182 became ill, and 29 died. An additional 39 cases, of which 5 were fatal, were reported among persons who had been near the hotel in that period. Subsequently, in 1977 and 1978, there were clusters of documented LD cases from Dallas, Texas; and in Columbus, Ohio; Burlington, Vermont; Kingsport, Tennessee; Nottingham, England; Los Angeles, California; Bloomington, Indiana; Atlanta, Georgia; New York, New York; Memphis, Tennessee; and Norwalk, Connecticut.

As methodology for the diagnosis of Legionnaires' disease has developed, it has become possible to detect clusters of cases that might otherwise have gone unrecognized. In some instances, LD has continued to affect people in localized areas without evidence of the temporal clustering that would suggest a point-source epidemic. A special instance of such areas of high endemicity is seen in several clusters of nosocomial LD. Although the LDB can infect persons who have no serious underlying illness, LD has frequently been associated with patients who have impaired immunologic defenses. Thus, cases may be associated with a given hospital only because the institution is a focal point for patients with renal heterografts or advanced malignancy. In some instances, though, hospitals, like other public buildings appear to have been the site of acquisition of infection with the LDB.

A characteristic of the epidemiology of LD is an association between infection and reservoirs of the LDB in the inanimate environment. The bacterium has been isolated from water from cooling towers or evaporative condensers in air-conditioning systems in Atlanta, Bloomington,

Columbus, Dallas, Los Angeles, Memphis, New York, Norwalk, and Pontiac, and from water and soil from streams in Atlanta and Bloomington. Success in recovering the organism from environmental sites does not implicate such sites in transmission of disease, but in some instances epidemiologic evidence implicating an environmental source has been supported by bacteriologic results.

More than 500 sporadic cases of LD have been reported to the Center for Disease Control (CDC) from at least 43 states and the District of Columbia (Figure 1). Approximately 19% of the reported cases were fatal, but this figure may overestimate the true proportion of fatal cases. The distribution of reported cases indicates a 2.4:1 ratio of males to females, and 90% of reported cases occur in people over 30 years of age. Cigarette smoking and alcohol abuse are clearly risk factors in acquiring LD, and a significant minority of patients report recent travel or exposure to excavation sites. Outbreaks and sporadic cases in the United States appear to be most common in the summer.

The Conference of State and Territorial Epidemiologists has collaborated with CDC in establishing a reporting system for this disease in the United States. Outside the United States, indigenous or imported cases of LD have been reported from Australia, Austria, Canada, Denmark, England, Israel, Italy, the Netherlands, Scotland, Spain, and Sweden. Cases have also been associated with travel to other European countries.

For some outbreaks and most sporadic cases, the mode of transmission of LD has not been documented, but in several outbreaks there is strong evidence for airborne spread of the agent, and it is possible that the airborne route is the only significant mode of transmission. Person-to-person spread of LD has not been conclusively demonstrated and must occur rarely if at all.

The need to isolate hospitalized patients with LD is not established, and superfluous isolation of patients is detrimental to their care. On the other hand, the presence of the LDB has been demonstrated in respiratory secretions from some infected persons; patients with LD may

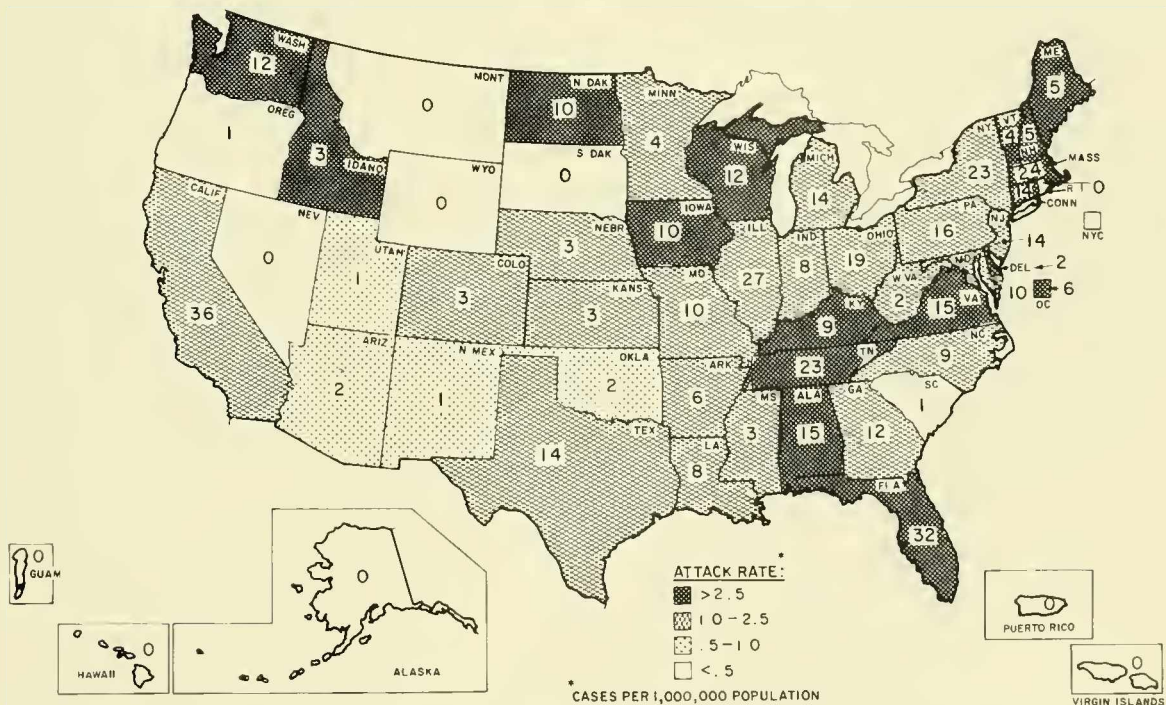


FIGURE 1. Sporadic cases of Legionnaires' disease in the United States, May 1973 through October 1978. Numbers within states indicate total reported cases per state.



receive assisted ventilation, with the attendant generation of aerosols; and other hospitalized patients may be particularly susceptible to infection with the LDB. Until more data are available to assess the risk of interpersonal spread of LD in the hospital, CDC recommends that patients with documented cases of LD be placed in respiratory isolation until they respond to antibiotic therapy. CDC further recommends that the nursing care for any patient with bacterial or viral pneumonia include oral secretion precautions. Isolation of patients with pneumonia should be limited to instances in which there is clinical or laboratory evidence of infection with certain transmissible respiratory pathogens (see *Isolation Techniques for Use in Hospitals*, 2nd ed., 1975, HEW Publication No. [CDC] 78-8314).

In practice, most cases of LD are diagnosed retrospectively, and the issue of isolation does not arise.

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# **PART TWO**

## **The Bacterium**





# Cultural and Biochemical Characterization of the Legionnaires' Disease Bacterium

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# Cultural and Biochemical Characterization of the Legionnaires' Disease Bacterium

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Although few cultural and biochemical characteristics have been defined, they are sufficiently distinct to enable a clinical microbiologist to differentiate the Legionnaires' disease bacterium (LDB) from other bacteria. Over 50 strains of LDB have been isolated and identified with the tests described here.

## CULTURAL CHARACTERIZATION

Inoculate the suspected isolate of LDB on charcoal yeast extract (CYE), Feeley-Gorman (F-G), and blood agar (BA) media (4). In addition, the medium on which the LDB first grew, Mueller-Hinton agar supplemented with 1% hemoglobin and 1% IsoVitaleX (MH-IH), may be inoculated (optional). (The chapter by Feeley et al. in this manual describes these media and their formulation.) Streak the inoculum on each medium to obtain well-isolated and discrete colonies.

The LDB grows best under aerobic conditions or in air plus 2.5% CO<sub>2</sub>, depending on the medium used. It does not grow anaerobically. Incubate all media except CYE at 35°C either in air plus 2.5% CO<sub>2</sub> or in a candle extinction jar. Incubate CYE agar at 35°C aerobically in a moist chamber. Examine all plates daily for 2 wk.

### A. Colonial Morphology

On the MH-IH agar, the LDB grows slowly. The colonies of the various strains which have not been transferred more than a few times on the agar are pinpoint in size at 3 days. By 5-7 days, well-isolated colonies may reach a diameter of 3 or 4 mm. The colonies are convex, circular, and have an entire edge. They are gray and glistening. In confluent areas the growth appears to be slightly moist.

On CYE agar growth of the LDB is apparent in 48 h or less in confluent areas at 35°C and 42°C. More growth occurs at 35°C than at 42°C. At 25°C, growth is apparent in 4-7 days. Further description of growth on F-G and CYE agars is in the chapter by Feeley et al.

### B. Microscopic Morphology

Remove a portion of the growth from the CYE agar with a bacteriologic needle. Emulsify the growth in approximately 1.0 ml sterile distilled water to make a slightly turbid cell suspension. Transfer a small aliquot of this suspension to each of two microscope slides, spread into a film, let air dry, and then heat fix. Stain the smears using the Gram and fat stain procedures below.

#### 1. Gram stain

Flood smears with crystal violet solution for 1 min; rinse with water. Add Gram's iodine for 1 min; rinse and decolorize with 95% ethyl alcohol. Counterstain either with



Figure 1. Gram stain using carbol fuchsin instead of safranin for the counterstain (LDB strain Philadelphia 1 from CYE agar).

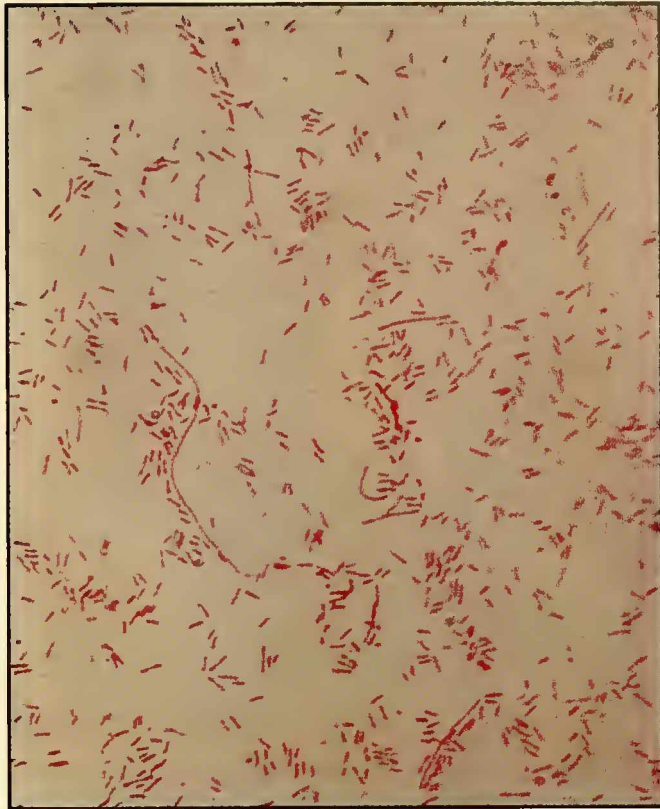


Figure 2. Gram stain using carbol fuchsin counterstain (LDB strain Knoxville 1 from CYE agar).

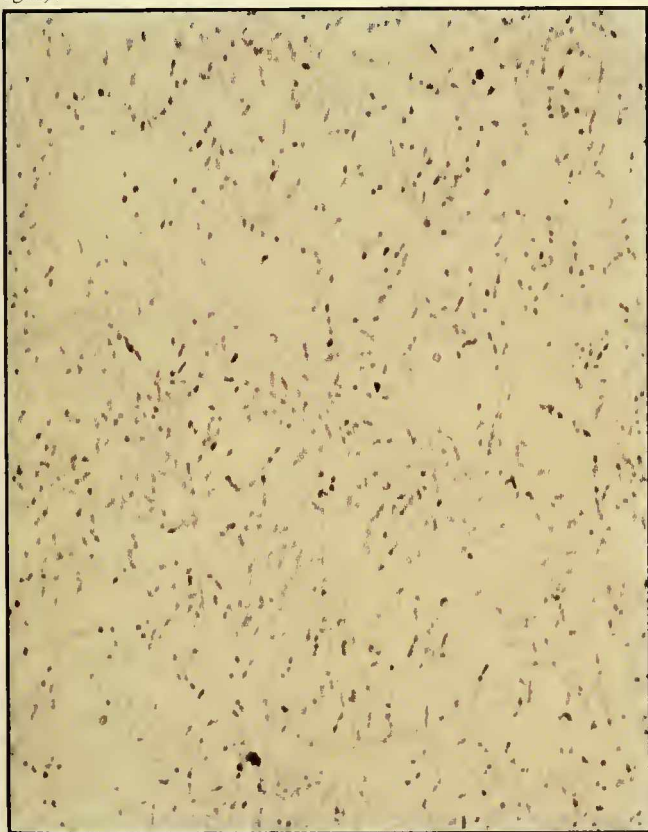


Figure 3. Fat stain of LDB.

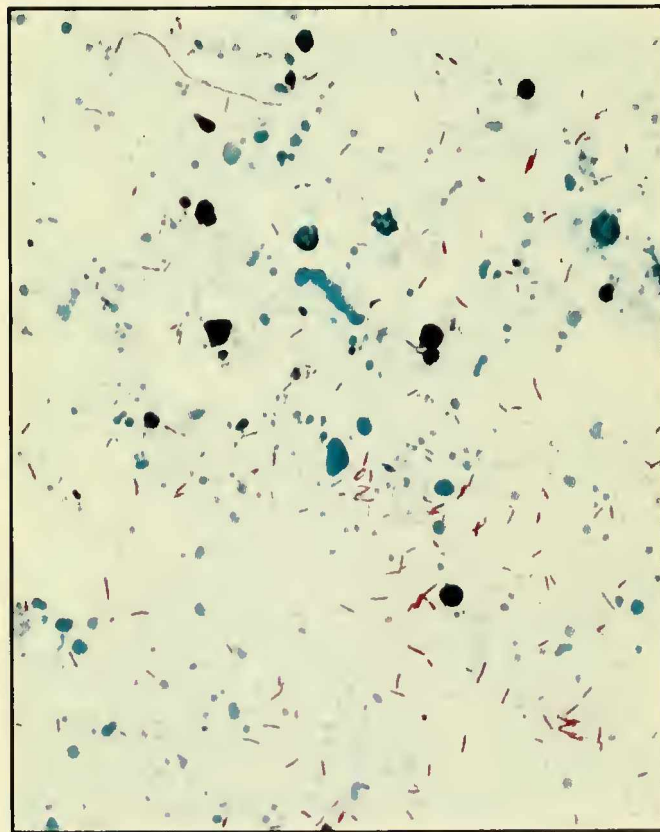


Figure 4. Giménez-stained smear of an LDB-infected egg yolk sac, courtesy of Dr. Joseph E. McDade.



safranin or carbol fuchsin for 1 min; rinse and air dry. Examine microscopically using oil immersion.

The LDB is a gram-negative bacillus having an approximate width of 0.5-0.7  $\mu\text{m}$  and a length that varies from 2 to 20  $\mu\text{m}$  or more. The cells appear red when counterstained with carbol fuchsin (Figs. 1 and 2) and light pink when counterstained with safranin. LDB cells on initial culture and passage on MH-IH medium may appear slightly swollen in regions having vacuoles that are stainable with the Sudan black B fat stain.

Prepare the carbol fuchsin counterstain by mixing the two following solutions.

#### Solution A:

Basic fuchsin	0.3 gm
Ethyl alcohol	10.0 ml

#### Solution B:

Phenol (melted crystals)	5 ml
Distilled water	95 ml

### 2. Fat stain

Flood heat-fixed smear with Sudan black B solution and allow to stand 5 to 15 min. Drain slide and blot dry. Apply xylene to smear several times either drop by drop or by immersing the slide into a staining jar containing xylene. Blot the smear dry and counterstain with safranin for 60 sec. Rinse with water and let air dry. Examine using oil immersion (Fig. 3).

Cells should appear pink and contain either blue-black or blue-gray droplets in areas that were vacuolated in the Gram stain.

Prepare the Sudan black B solution by dissolving 0.3 gm of Sudan black B in 100 ml of 70% ethyl alcohol and letting the solution stand overnight at room temperature.

### 3. Giménez stain

The LDB can be demonstrated in an infected egg yolk sac with the Giménez stain; the LDB stains red, and the yolk sac tissue appears as blue-green background cells (Fig. 4). The Giménez reagents and procedure are described in the chapter by McDade. This stain can also be used for impression smears and frozen sections (see chapter by Blackmon et al. in this manual).

### 4. Flagella stain

Recently, flagella have been observed (Berenice M. Thomason, personal communication) on several strains of the LDB by using a modified Leifson flagella stain (3) or a simplified silver-plating stain for flagella (9).

## C. Preliminary Identification

Organisms that have the three characteristics described below and have a cut-glass appearance on F-G agar should be strongly suspected of being LDB, and should be further examined by procedures listed under Definitive Identification.

### 1. Requirement for L-cysteine-HCl

All strains of LDB examined to date require L-cysteine-HCl for growth. Therefore organisms that grow on BA medium, which does not contain L-cysteine-HCl, are unlikely to be LDB. Gram-negative bacilli that grow only on L-cysteine-HCl-containing media such as CYE, F-G, and MH-IH agars are possibly LDB. Examine these further for brown pigment and yellow fluorescence production.

## 2. Brown pigment

Inoculate either an F-G agar plate or slant, and incubate at 35°C in air plus 2.5% CO<sub>2</sub>. Pigment should be observable in areas of heavy confluent growth after 2-3 days of incubation. Browning may also be produced with other media enriched with L-tyrosine at a concentration equivalent to that found in Mueller-Hinton agar (1). Browning is not observable with CYE agar.

## 3. Yellow fluorescence

Inoculate a tube of F-G broth heavily with the suspected organism. Incubate at 35°C in air plus 2.5% CO<sub>2</sub>. Examine daily in the dark with a 366-nm ultraviolet light. Broth should fluoresce bright yellow within 3-4 days of incubation and continue to do so with additional incubation. F-G broth has the same formulation as F-G agar except that it lacks agar-agar, and the concentration of soluble ferric pyrophosphate (wt/vol) is 100 mg/liter. Tube broth in 2-ml aliquots in screw-capped tubes (13 x 100 ml).

## D. Definitive Identification

The LDB characteristically gives negative reactions for most biochemical tests with the exceptions of catalase, oxidase, and  $\beta$ -lactamase. Therefore, an isolate can only be definitively identified as LDB with results from the direct fluorescent antibody (FA) test, gas liquid chromatography (GLC), and deoxyribonucleic acid (DNA) hybridization.

### 1. Direct FA test

Prepare smears of the candidate organism, and stain it with all available conjugates according to the methods described by Cherry and McKinney in this manual.

### 2. Gas liquid chromatography

Prepare extracts of the candidate organism by the methods described by Moss et al. in this manual, and examine for cellular fatty acids.

### 3. Deoxyribonucleic acid (DNA) hybridization

Brenner et al. have used this test very effectively for studying the LDB. Their procedure is described in this manual. However, because this test is very specialized and time-consuming, it should be reserved for isolates that (1) have a compatible cellular fatty acid profile by GLC and do not stain with direct FA conjugates prepared against all established serogroups or (2) are considered to be LDB but have some unusual characteristic.

## BIOCHEMICAL CHARACTERIZATION

This section is for those investigators who wish to further characterize an isolate as the LDB.

### A. Carbohydrate Utilization

The LDB will not grow in the commonly used carbohydrate test media. For this reason, carbohydrates were incorporated into especially prepared basal media to demonstrate utilization by the LDB. All 15 strains tested grew but did not produce acid (Riley, Weaver, and Feeley, unpublished data). Similar results were obtained with "rapid sugar" techniques (2). One test that can be easily performed is *Starch Utilization*. Heavily inoculate an area of an F-G agar plate and incubate for 3-5 days at 35°C in air plus 2.5% CO<sub>2</sub>. Flood plate with Gram's iodine and observe. Most of the agar should stain blue because of the reaction of the starch contained in F-G agar with the added iodine. There should be a clear zone void of blue color extending about 4 mm



beyond the edge of the bacterial growth. This reaction is considered to be a positive test and suggests that starch has been utilized. The LDB is positive by this starch utilization test.

## B. Catalase

Inoculate an F-G or CYE agar slant heavily; incubate at 35°C for 48-72 h. Examine the growth for catalase activity by adding 2 to 3 ml of 3% hydrogen peroxide to the agar slant. Bubbles should appear within 30 sec. The LDB catalase reaction is positive and can be speeded up if the growth is "broken up" slightly with a sterile capillary pipette. The "sticky" consistency of the growth apparently reduces contact between the peroxide and the LDB cells.

## C. Gelatinase

This test can be performed with a photographic film or a gelatin cupule. Prepare a turbid suspension in broth (Mueller-Hinton or heart infusion) by emulsifying some growth from an F-G agar plate.

### 1. Film method

Place a strip (0.5 x 1.5 cm) of photographic film (Kodak, plus-X) into 0.5 ml of the turbid cell suspension in a screw-capped tube (13 x 100 mm). Incubate at 35°C in air plus 2.5% CO<sub>2</sub>. The emulsion on the film should be digested within 48-72 h if the isolate is the LDB.

### 2. Cupule method

Inoculate an API (Analytab Products Inc.) gelatin cupule with the turbid cell suspension. Incubate at 35°C in air plus 2.5% CO<sub>2</sub>. Gelatin should be digested within 48-72 h if the isolate is the LDB.

## D. $\beta$ -Lactamase

Inoculate the test organism heavily on the medium developed by Martin and Lewis (5) to detect penicillinase-producing *Neisseria gonorrhoeae*. Incubate at 35°C in air plus 2.5% CO<sub>2</sub> for 7 days. Although the LDB will not grow well on this medium, it will inactivate the penicillin contained in the medium and allow growth of the *Sarcina lutea* that is also in the medium. Growth of the *S. lutea* indicates lactamase activity by the test organism. All LDB identified to date produce  $\beta$ -lactamase. In addition,  $\beta$ -lactamase is a consistent characteristic of all LDB (7) examined with the chromogenic cephalosporin test (see Appendix).

## E. Nitrate

Potassium nitrate (0.2%) is incorporated into agar slants in which Fildes' enrichment is substituted for hemoglobin. (Reduction of nitrate by *Pseudomonas aeruginosa* and *Yersinia enterocolitica* can be readily demonstrated with the medium). The slants are inoculated heavily and are incubated for 7 days. All LDB isolated to date are nitrate negative.

## F. Oxidase

Wet a piece of filter paper with a 0.5% solution of oxidase reagent (6) (tetramethyl-p-phenylenediamine dihydrochloride) which has been freshly prepared or refrigerated for less than a week. Test the suspect isolate for oxidase activity by transferring some growth from an F-G agar plate with a platinum loop; rub the growth into the previously prepared filter paper. A dark blue color should appear in 9-10 sec, indicating a weakly positive test if the isolate is the LDB.

## G. Urease

Inoculate a Christensen's urea agar (S) slant heavily with the suspected isolate. Incubate at 35°C in air plus 2.5% CO<sub>2</sub> for 24 h. The slant should not change color if the isolate is the LDB, since all LDB isolated to date are urease negative by this test. The LDB does not grow on this agar; however, if it were urease positive, the heavy inoculum would contain a sufficient amount of the enzyme to cause a positive reaction.

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# Physiology: Characteristics of the Legionnaires' Disease Bacterium in Semisynthetic and Chemically Defined Liquid Media

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# Physiology: Characteristics of the Legionnaires' Disease Bacterium in Semisynthetic and Chemically Defined Liquid Media

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## INTRODUCTION

Recently, semisynthetic and chemically defined liquid media were described (6) for growing the Legionnaires' disease bacterium (LDB). These media have allowed certain new observations concerning growth characteristics of the organism and verification of characteristics observed using the more complex Mueller-Hinton medium supplemented with hemoglobin and IsoVitaleX, Feeley-Gorman (F-G) agar, and charcoal yeast extract agar (CYE) (1, 2). In this chapter, we discuss the media, the methods used for determining growth responses, certain idiosyncrasies related to the use of the media, and some nutritional requirements and physiological aspects of the LDB.

## METHODS

### A. Media

The basic formula for the semisynthetic medium for growing the LDB is in Table 1; that for the chemically defined medium is in Table 2. A 2X concentrated broth medium was prepared by combining double-strength solutions, adjusting the pH, and sterilizing by filtration (0.45 micron, Millipore Corp.). The other solutions, which contained starch and oleic acid or agar, or both, were prepared, autoclaved for sterilization, and added to the 2X broth medium while they were hot to produce either the complete medium or a medium of 5/4 or greater concentration. Liquid media were used within 24 h. Complete agar media were tubed at 10 or 15 ml; 8 and 12 ml were used if they were at a 5/4 or greater concentration. These tubes or other basal agar media were stored at 5°C for 1 to 2 wk. Before they were used, agar media were melted by being steamed for 5 min; if necessary, test materials were added to the melted agar. The melted media were then poured into plates, which were always used within 16 h. Similarly, culture tubes (18 X 150 mm) were prepared by adding 1 ml of water or chemical solutions to 4 ml of 5/4-strength basal medium.

### B. Standard Inocula

Most of our early studies (6) were done with LDB strain Philadelphia 1, which was isolated from a patient who died in the 1976 epidemic of Legionnaires' disease (LD) in Philadelphia (3). We later included other strains in all stages of media evaluation. Inocula for various experiments were prepared as follows: cultures were grown on F-G plates or slants; cells were washed off the agar surface with sterile distilled water and stored as thick suspensions (approximately 9.0 mg dry wt of cells/ml) at -70°C. Media were inoculated with a freshly thawed cell suspension by placing 1-3 drops (0.05 to 0.15 mg dry wt cells) on agar plates containing 10 or 15 ml of medium or in tubes (18 X 150 mm) containing 5 to 15 ml of broth. In broth, this inoculum was sufficient to raise the zero time suspensions to an absorbance (A) of 0.05 (1 drop or 0.05 mg) to 0.20 (3 drops or 0.15 mg) at 660 nm. Relationship of A to cellular dry weight was determined by suspending



Table 1. Semisynthetic medium (SSLp) for growth and maintenance of the Legionnaires' disease bacterium (5)

**PART I: Casein Hydrolysate-Vitamin Base**

Steps and solutions for preparing medium (in sequence):

1. **Salts (in grams):**

KH <sub>2</sub> PO <sub>4</sub> . . . . .	8.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> . . . . .	8.0
MgSO <sub>4</sub> ·7H <sub>2</sub> O . . . . .	0.86
CaCl <sub>2</sub> (anhydrous) . . . . .	0.08
ZnSO <sub>4</sub> ·7H <sub>2</sub> O . . . . .	0.89

  - a. Dissolve in 500 ml and make to 1 liter with distilled water.
  - b. Add 250 ml/liter of medium. Bring the 250 ml of solution to 350 ml with distilled water before adding other components to minimize formation of precipitate.
2. **Casein hydrolysate, 10% acid-hydrolyzed, vitamin-free solution; add 80 ml/liter.**
3. **Vitamin suspension (in milligrams):**

Inositol. . . . .	200
Thiamine hydrochloride . . . . .	200
Calcium pantothenate . . . . .	200
Riboflavin . . . . .	200
Nicotinamide . . . . .	100
Biotin. . . . .	10

  - a. Suspend and make to 1 liter in distilled water.
  - b. Vitamin suspension can be stored at 5°C. Add a few drops of chloroform as preservative. Shake the flask and remove an aliquot from the top.
  - c. Add 10 ml/liter of medium.
4. **DL-thioctic acid:**
  - a. Dissolve 10 mg of DL-thioctic acid in 10 ml of 95% alcohol; store at -20°C.
  - b. Add 0.1 ml/liter.
5. **Coenzyme A:**
  - a. Dissolve 10 mg of coenzyme A in 10 ml distilled water.
  - b. Add 2 drops of 0.05% Na<sub>2</sub>S·5H<sub>2</sub>O solution made in freshly boiled distilled water. Store at -20°C.
  - c. Add 0.1 ml/liter of medium.
6. **Solid additions (in grams):**

α-ketoglutaric acid . . . . .	1.00
L-cysteine-HCl. . . . .	0.50
Glutathione (reduced) . . . . .	0.50
L-asparagine . . . . .	0.10
L-tryptophane. . . . .	0.02

7. **Minor elements (in grams):**

- a. Add 1.0 ml of concentrated HCl to 100 ml of distilled water.
- b. Dissolve each component in the order listed before adding next component.
 

FeSO <sub>4</sub> ·7H <sub>2</sub> O . . . . .	5.70
MnCl <sub>2</sub> ·6H <sub>2</sub> O . . . . .	0.80
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O . . . . .	0.15
- c. Make to 1000 ml with distilled water.
- d. The minor elements can be stored at room temperature. Should oxidation of Fe<sup>++</sup> occur, as evidenced by red precipitate, make a new solution.
- e. Add 10 ml/liter of medium.

8. **Adjust pH to 6.3 with 20% KOH.**9. **Hemin:**

- a. Suspend 200 mg hemin in 10 ml distilled water and bring into solution by adding 2 drops of concentrated NH<sub>4</sub>OH.
- b. Make to 100 ml with distilled water.
- c. Add 1 ml/liter of medium.

10. **Neutralize carefully to pH 6.50 with 20% KOH.** (NOTE: As pH approaches 6.5, the solution will turn purple black, as a cysteine-Fe complex forms. This color disappears after the solution stands. Filtering medium containing a small amount of precipitate has not affected growth.) Bring volume to 500 ml, and sterilize by filtering through a 0.45-μm membrane filter (Millipore) to give 2X concentrated basal solution.

**PART II: Starch-Oleic Acid Solution**1. **Starch:**

Suspend 0.5 g of soluble starch (Eastman, Baker, Mallinckrodt, ACS) in 50 ml of distilled water and pour into 450 ml of boiling distilled water.

2. **Oleic acid:**

- a. Suspend 100 mg of oleic acid in 50 ml distilled water, and carefully bring into solution by titrating with NaOH to pH 7.0 with 1 drop of 0.05% phenol red. Warm if necessary.

b. Adjust to final volume of 100 ml.

3. **Add 1 ml of the oleic acid solution to the 500-ml starch solution. Autoclave starch-oleic acid solution.**

**PART III: Final Medium**1. **Broth medium: one liter:**

Combine 500 ml of casein hydrolysate-vitamin base (filter sterilized) with 500 ml starch-oleic acid solution (autoclaved) to prepare 1 liter of semisynthetic (SSLp) broth medium.

2. **Agar medium: one liter:**

- a. Prepare 500 ml casein hydrolysate-vitamin base as above.
- b. Prepare 500 ml starch-oleic acid-agar solution:
  1. Suspend 2 g of soluble starch instead of 0.5 g.
  2. Add 10 ml of the 1 mg/ml oleic acid solution instead of 1 ml.
  3. Add 15 g agar.
  4. Autoclave.
- c. Combine the two solutions and pour plates or slants.

The medium is to be used in growth conditions including a CO<sub>2</sub> seal per tube (or incubation under similar conditions of increased CO<sub>2</sub> tension) and moderate shaking conditions. Good but slower growth may be obtained in static tubes with seals, if the culture tube with 5 to 20 ml of medium is incubated in a near-horizontal position. Increased CO<sub>2</sub> tension on agar plates is not required, but the plates should be incubated in a humidity chamber. To prepare the CO<sub>2</sub> seal for tubes, trim off the cotton plug extending beyond the glass wall. Push this short plug into the tube to an inch below the lip of the tube, and place a small wad of absorbent cotton on top of the plug. Add 0.3 ml of 10% Na<sub>2</sub>CO<sub>3</sub> to each wad of absorbent cotton. Add 0.3 ml of 1 M KH<sub>2</sub>PO<sub>4</sub> to the same spot, and close immediately with a rubber stopper. If a series of tubes is to be prepared, the Na<sub>2</sub>CO<sub>3</sub> can be added to all, after which add KH<sub>2</sub>PO<sub>4</sub> solution to three tubes before sealing them with rubber stoppers. To open seal, remove rubber stopper and absorbent cotton, flame tube, and withdraw cotton stopper as desired.

washed cells in 1% Formalin, measuring the A, drying aliquots for 48 h at 75°C, and recording the dry weight (Fig. 1). All A's were measured in a model B Beckman spectrophotometer at 660 nm.

### C. Incubation

For maintenance, cultures were incubated in candle jars or in 95% air-5% CO<sub>2</sub>. Experimental agar cultures were incubated in air in moist chambers, in moist candle jars, in tightly sealed chambers dried and depleted of CO<sub>2</sub> by sandwiching the agar plates between open petri dishes filled with NaOH pellets, in dessicators from which CO<sub>2</sub> was depleted by the presence of concentrated solutions of NaOH, or in tightly sealed containers with an atmosphere containing approximately 4.8% CO<sub>2</sub>. The CO<sub>2</sub> in sealed containers was generated by mixing 2 ml of 10% Na<sub>2</sub>CO<sub>3</sub>/liter of internal volume with an excess of 2.5 N sulfuric acid. Growth on agar plates was measured by adding 5 ml of distilled water or 1% Formalin to each plate and removing the cells from the surface of the closed agar plate with a 1-cm wire brad cut from a paper clip. The wire brad was placed on the agar and rotated by placing the plate on a magnetic stirring device. The

**Table 2**  
**Composition of a chemically defined medium (CDLp) for the**  
**Legionnaires' disease bacterium**

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#### Amino acid-organic acid solution

Add 5 g each of serine, alpha-ketoglutaric acid, and sodium pyruvate to 500 ml of water. Add 0.5 g of each of the 18 amino acids listed below and bring the final solution to 1 liter.

L-alanine, L-aspartic acid, L-asparagine (monohydrate), L-arginine (monohydrochloride), L-histidine HCl (monohydrate), L-isoleucine, L-glutamic acid, L-glutamine, glycine, L-leucine, L-lysine (monohydrochloride), L-methionine, L-phenylalanine, L-proline, L-threonine (allo-free), L-tryptophane, L-tyrosine, and L-valine.

#### Basal medium

1. Combine the following ingredients in the order shown:  
 250 ml of the 4X salts solution, Table 1.  
 200 ml of the amino acid-organic acid solution described above.  
 10 ml of the vitamin solution, Table 1.  
 0.1 ml of the thioctic acid solution, Table 1.  
 0.1 ml of the coenzyme A solution, Table 1.  
 0.5 g L-cysteine HCl.  
 0.5 g glutathione (reduced).  
 10 ml of the minor element solution, Table 1.
2. Adjust pH to 6.3 with 20% KOH.
3. Add 1 ml of the hemin solution, Table 1.
4. Adjust the pH to 6.5 with 20% KOH, bring the volume to 500 ml with distilled water, and sterilize this 2X concentrated medium by filtering through a 0.45- $\mu$ m membrane filter.

#### Final medium

Bring the sterile 2X concentrated medium to final volume with sterile distilled water. For general use, the final broth medium should contain 0.05% soluble starch (Eastman, Mallinckrodt, or Baker), and an agar medium should contain 1% "Ion Agar" No. 2 (oxoid) and 1% soluble starch.

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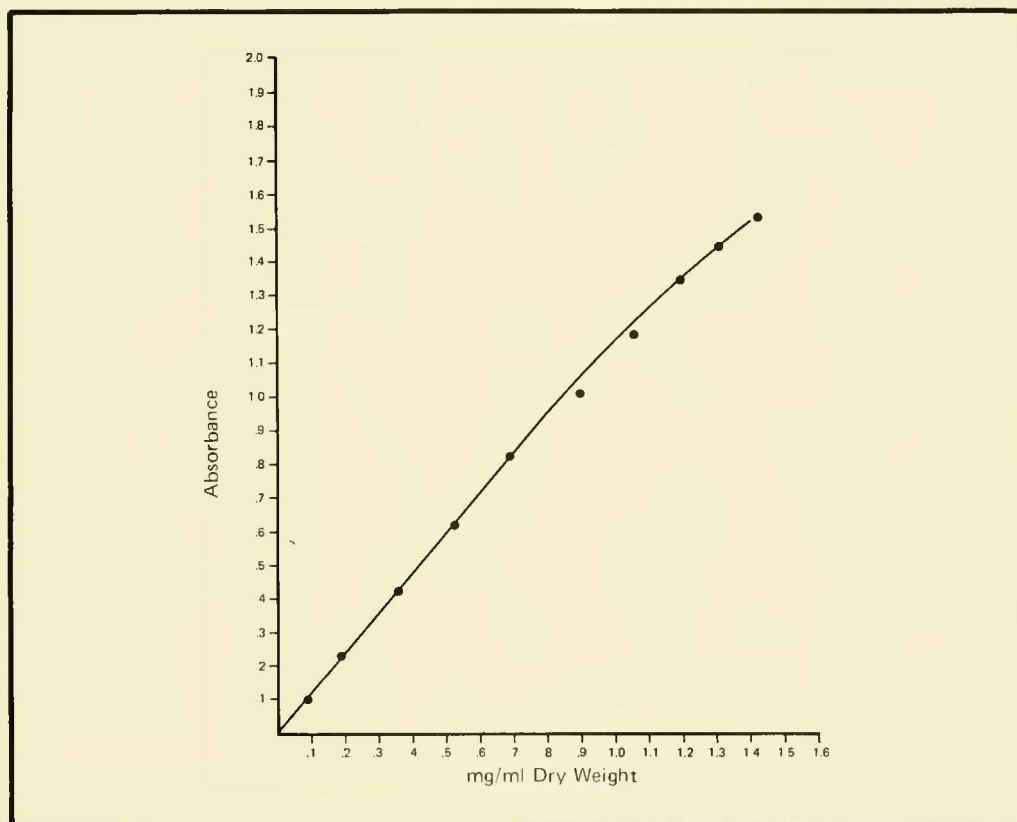
worker is safe from aerosols when removing cells with the spinning wire brad, and the procedure does not release agar into the cell suspension (4). The suspensions were drawn off and diluted to absorbance readings of 1.0 or less at 660 nm.

Anaerobic, aerobic, aerobic without  $\text{CO}_2$ , or aerobic + increased  $\text{CO}_2$  cultural environments were produced in test tubes with absorbent cotton plugs and combinations of saturated pyrogallol, 20% KOH, 10%  $\text{Na}_2\text{CO}_3$ , and 1 M  $\text{KH}_2\text{PO}_4$ ; rubber stoppers were then inserted in the tops of the tubes (9). Virtually all of the broth cultures were incubated with  $\text{Na}_2\text{CO}_3$ - $\text{KH}_2\text{PO}_4$  ( $\text{CO}_2$ ) seals. In general, air with increased  $\text{CO}_2$  tension was created by adding 0.3 ml each of 10%  $\text{Na}_2\text{CO}_3$  and 1 M  $\text{KH}_2\text{PO}_4$  to the cotton plug. The test tubes were placed on a rotary shaker at 70 rpm at  $37^\circ\text{C}$ ; A's were read at chosen intervals. Other commonly used bacteriological, serological, or chemical methods have been described (6).

## RESULTS

### A. Determination of basal growth conditions and development of a basal medium.

In the initial "probing" experiments, we examined culture conditions and media formulations which would support consistent growth of small inocula and provide maximal cell density. The experience of others and our own experimental findings showed that the major factors which affect growth of the LDB are size and condition of the inoculum and the presence of air, carbon dioxide, pyruvate, alpha-ketoglutaric acid, and cysteine. Certain of these results are shown below.



**Figure 1.** Relationship of dry weight to absorbance (660 nm) of the inoculum of Legionnaires' disease bacterium strain Philadelphia 1, grown on the F-G medium. Cells used were obtained from CYE agar and were singles or doublets of small, cigar-shaped bacilli.

The semisynthetic medium (Table 1) generally supported excellent growth of the standard inocula described, although inocula with A's less than 0.10 very often did not grow when initially transferred to this medium. However, cells harvested from the primary passage to the semisynthetic medium grew when transferred at lower cell densities. Growth on the chemically defined medium occurred readily on the first transfer, but with subsequent passages growth occurred sooner and reached higher densities on the chemically defined medium than on the semisynthetic medium.

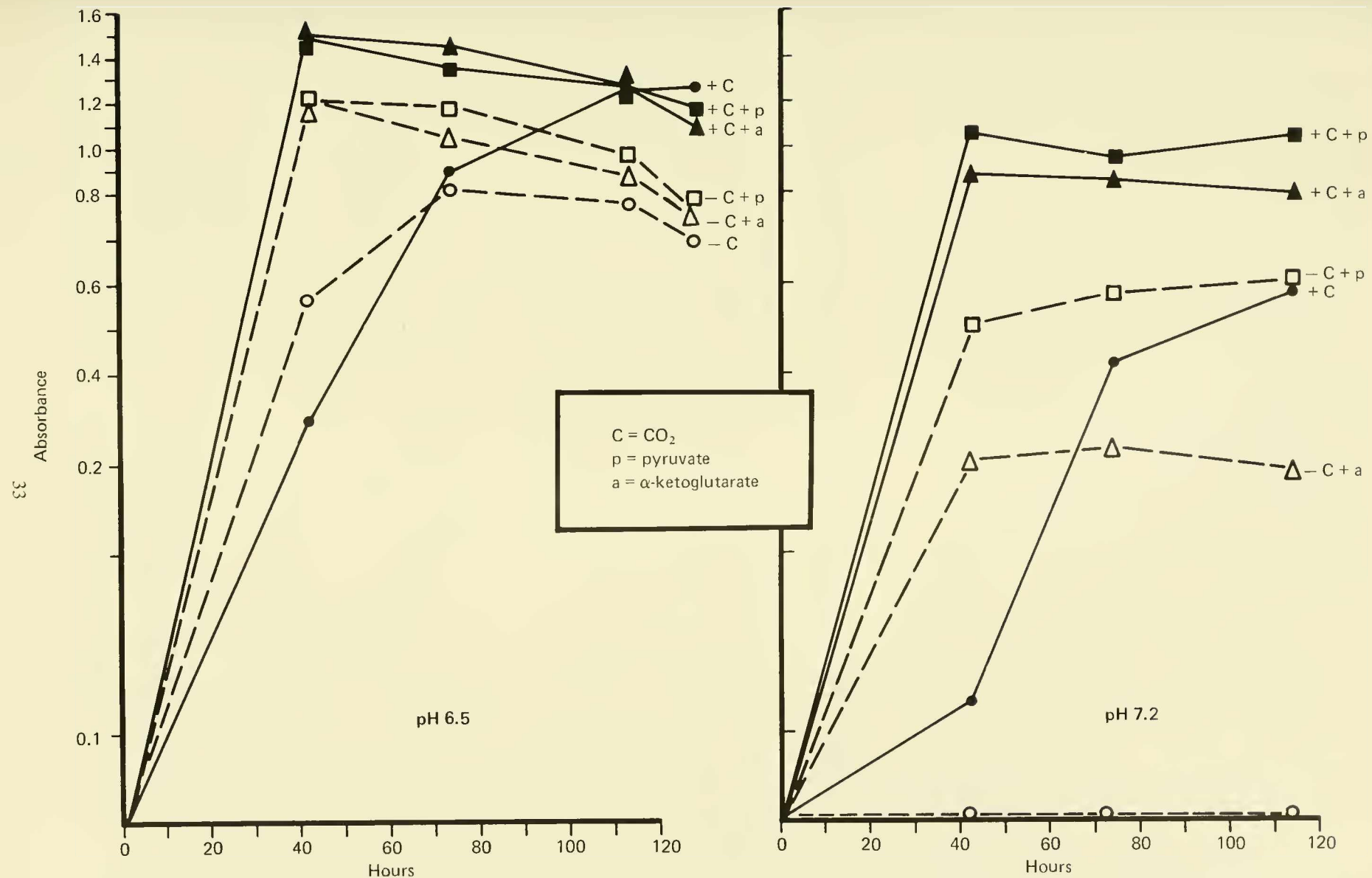
In an experiment with the semisynthetic broth in flasks, in shaking tubes containing various volumes of liquid (5 to 15 ml), and under different conditions of aerobiosis induced by incubation with or without shaking, growth was obtained in tubes which were incubated with shaking and had CO<sub>2</sub> seals. No growth was observed in tubes incubated anaerobically. Tubes incubated stationary with CO<sub>2</sub> seals did not contain significant growth until placed on the shaker, after which their A's increased from 0.19 to 0.77. Tubes incubated stationary without CO<sub>2</sub> seals had no visible growth for several days but had final A's ranging from 0.48 to 0.61 after CO<sub>2</sub> seals were added and the tubes were again incubated with shaking. These results suggested that the LDB was a strict aerobe, which required CO<sub>2</sub> and ready access to oxygen, and that it was potentially sensitive to excessive aeration.

Adding agar to the chemically defined medium strongly inhibited growth of low concentrations of inocula. Dilutions of inocula ranging from 10<sup>-1</sup> to 10<sup>-7</sup> grew in 7 to 10 days when "plated" in broth in petri dishes and incubated in a 95% air-5% CO<sub>2</sub> atmosphere. Similar plates containing Difco agar had no apparent growth at dilutions of more than 10<sup>-2</sup>. Others with 1% Ion Agar No. 2 supported growth at 10<sup>-4</sup>, but when 1% soluble or insoluble purified potato starch or 0.2% charcoal (Norit A) was added, the effect of the agar was completely reversed. The semisynthetic agar and the F-G agar under moist, aerobic conditions supported comparable levels of growth and cell yields, although the lag period on the semisynthetic medium was shorter. In air, with a pH above 6.8, the growth rates (but not final cell yields) were definitely lower than those obtained in candle jars. However, 0.1% alpha-ketoglutaric acid strongly increased the growth rate, and cell yields were substantially higher at pH's above 6.8. Higher cysteine concentrations had no apparent effect. The conditions required for growth raised many questions about the role of pH and CO<sub>2</sub>, the depletion of oxygen in a candle jar, the use of alpha-ketoglutaric acid as a source of CO<sub>2</sub>, and the effect of pH on cysteine in the medium.

These data did not clearly delineate the functions of the factors tested. More complete experiments were conducted in air, air with CO<sub>2</sub> added, and air with CO<sub>2</sub> removed, using media containing low concentrations of casein hydrolysate. The effects of pyruvate and alpha-ketoglutarate were also determined (Fig. 2). The results obtained in "air" were between the extremes obtained with "added" and "removed" CO<sub>2</sub> and are not shown in Fig. 2. At pH 7.2, no apparent growth occurred in air or in air depleted of CO<sub>2</sub> when neither pyruvate nor alpha-ketoglutaric acid was added. The results also showed that at pH 7.2 without CO<sub>2</sub>, alpha-ketoglutaric acid and pyruvate stimulated rates of growth and cell yields; however, the resulting cell yields were not greater than 33%-66% of that obtained when only CO<sub>2</sub> was added. Carbon dioxide was clearly required for growth at pH 7.2 and organic acids could not entirely replace it. Further, at this pH, the growth rates and cell yields were significantly higher in the presence of pyruvate than in that of alpha-ketoglutarate.

However, at pH 6.5 with no organic acids added, CO<sub>2</sub> improved but was not required for growth (Fig. 2). When the organic acids were present, cell yields in the absence of CO<sub>2</sub> were only 66% of those obtained when CO<sub>2</sub> was present. In the presence of 5% CO<sub>2</sub> for an extended growth period, the cell yields without added organic acids were only 85% of those obtained with added pyruvate or alpha-ketoglutarate. These results indicate at least two separate roles for CO<sub>2</sub> and the organic acids and emphasize that both are required for maximal growth.





**Figure 2.** Effect of CO<sub>2</sub>, 0.4% pyruvate and 0.4% alpha-ketoglutaric acid on the growth of Legionnaires' disease bacterium on modified semisynthetic agar (only 0.4% casein hydrolysate) at pH 6.5 and pH 7.2. Each point represents the average obtained with three plates. The plates were incubated in a moist air chamber, in air + 5% CO<sub>2</sub>, or in sealed dessicators with 40% NaOH in the bottom. The results obtained in the presence of air are not shown, but were between those obtained in the other atmospheric conditions, except that no growth was obtained at pH 7.2 in the absence of pyruvate or α-ketoglutaric acid.

In the liquid semisynthetic or chemically defined media, the standard inoculum will grow in dilutions of  $10^{-7}$  incubated 7 to 10 days in shallow layers in a  $\text{CO}_2$ -enriched atmosphere. At a dilution of  $10^{-2}$  or  $2 \times 10^{-2}$  (final A = 0.05), this inoculum will grow slowly in slanted tubes but will not produce the maximal cell yields obtained in control shake cultures. However, inocula of lower concentrations do not grow with shaking. Even when we steamed the broths for 5 min to lower the redox potential (as might occur with large inocula), growth of low concentrations of inocula was not stimulated by shaking. Without  $\text{CO}_2$  seals, there was less growth, and heavy brown pigments were formed which were associated with bacterium's stationary phase (Table 3). This effect of air curbing growth was directly correlated with a pH rise from 6.5 to 7.9 during incubation in air, whereas growth under the  $\text{CO}_2$  seal barely increased the pH from 6.35 to 6.45 (Table 3). Control studies showed that the  $\text{CO}_2$  seals decreased the initial pH from 6.5 to 6.2 within 48 h. These results show that in part the  $\text{CO}_2$  atmosphere functioned as a buffer to maintain the initial pH in agar media.  $\text{CO}_2$  may have also regulated metabolism.

Using reagent-saturated filter paper discs on the surface of semisynthetic agar, we observed no effects with succinate, fumarate, lactate, acetate, oxalate, gluconolactone, glucuronic acid, glycerol, gelatin, and cobalamin. Yeast extract, pyruvate, and alpha-ketoglutaric acid stimulated growth, whereas citrate strongly inhibited it.

## B. Determination of substrate

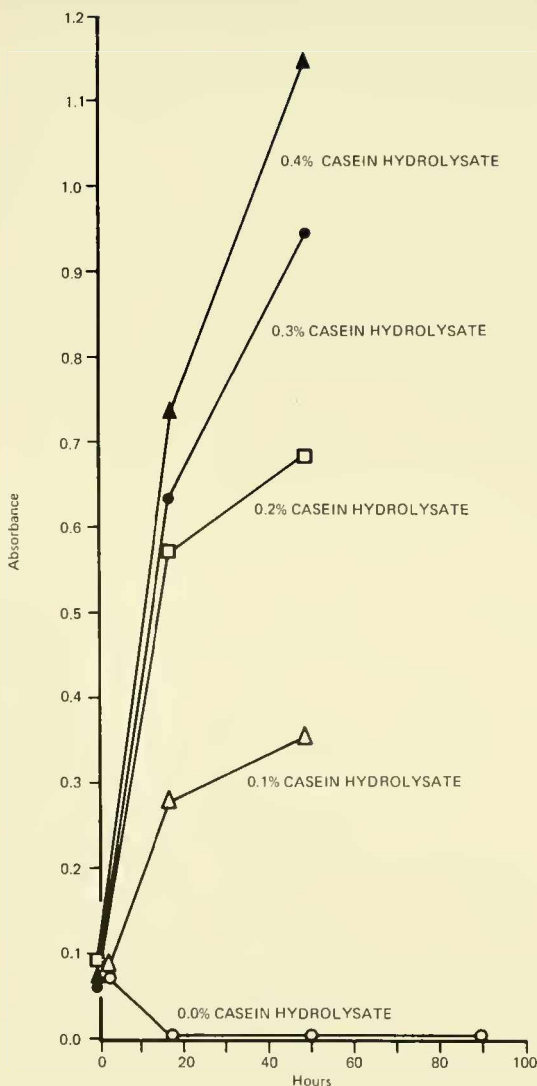
Having determined conditions of growth and developed a basal semisynthetic medium which satisfactorily supported growth, we examined the medium to detect components which acted as substrate sources of energy.

An examination of the semisynthetic medium for constituents which affect growth showed that cell yields were proportional to the concentration of casein hydrolysate (Fig. 3). Of the amino acids tested, the presence of histidine, threonine, tryptophane, tyrosine, or serine gave substantially increased cell yields, with serine causing the greatest increase (Fig. 4). Cell yields were slightly higher in the presence of glutamine, lysine, or proline; cell yields were not apparently affected by adding alanine, arginine, aspartic acid, cystine, glutamic acid, isoleucine, leucine, methionine, and/or valine to the casein hydrolysate. Chromatographic experiments did not show that any one amino acid disappeared during growth in 0.4% casein hydrolysate. These results show that the above specified amino acids were major sources of energy for growth.

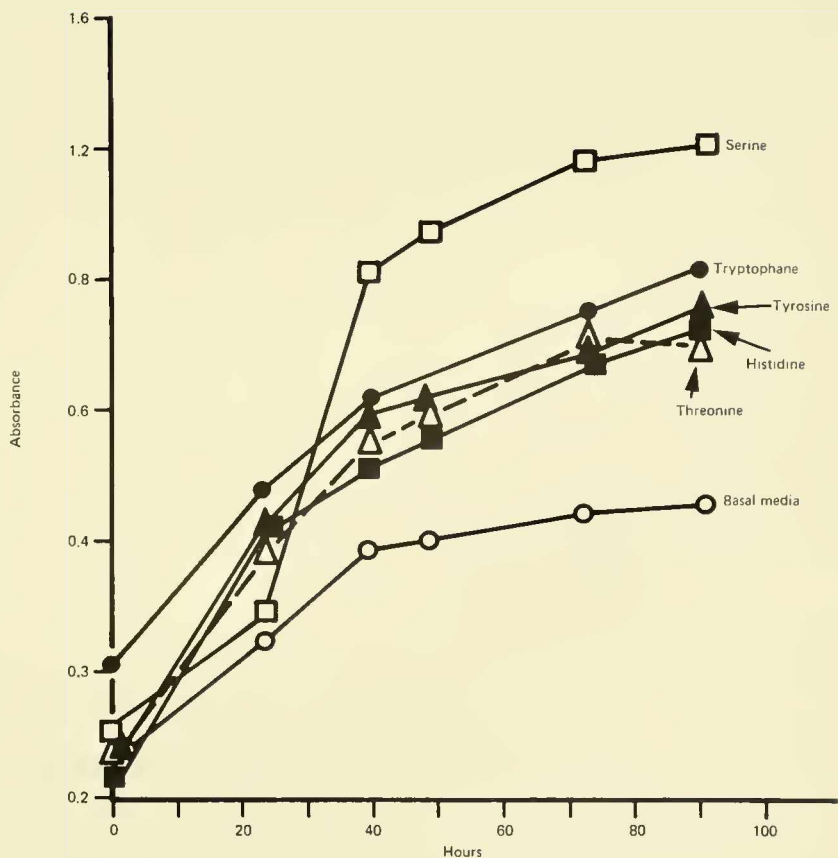
Table 3  
Effect of air and air + carbon dioxide on growth and final  
pH in shake culture\*

Initial A (inoculum)	Condition	Final A	Final pH	Description
0.05	air + $\text{CO}_2$	1.16	6.35	no color
0.10	air + $\text{CO}_2$	1.13	6.40	no color
0.15	air + $\text{CO}_2$	1.12	6.40	no color
0.05	air	.75	7.65	high melanin
0.10	air	.74	7.70	high melanin
0.15	air	.75	7.75	high melanin

\* Medium was the semisynthetic (SSLp) broth with 1.0% casein hydrolysate and an initial pH of 6.5. Cultures were incubated for 65 h at  $37^\circ\text{C}$  and shaken at 70 rpm. Carbon dioxide ( $\text{CO}_2$ ) was generated with 0.3 ml of 10%  $\text{Na}_2\text{CO}_3$  plus 0.3 ml 2.5 N  $\text{H}_2\text{SO}_4$  per tube.



**Figure 3.** Effect of casein hydrolysate concentration on growth rates and cell yields of the Legionnaires' disease bacterium. The basal medium was the SSLp broth without casein hydrolysate. CO<sub>2</sub> seals were used on tubes incubated at 37°C and shaken at 70 rpm.



**Figure 4.** The effect of single amino acids added to the basal SSLp medium containing 0.2% casein hydrolysate. CO<sub>2</sub> seals were used on tubes incubated at 37°C and shaken at 70 rpm.



On the other hand, the presence or absence of glucose in the semisynthetic medium had no effect upon growth. Although 1% glucose caused lactate to form in 0.8 to 0.9- $\mu$ M/ml amounts, no lactate was formed in the absence of glucose. No sugars other than glucose were tested. The presence of amylose, amylopectin, dextrin, glycogen, yeast extract, or bovine albumin did not affect cell yields.

In the presence of 0.1% alpha-ketoglutaric acid, some acids, including 0.4% pyruvate, fumarate, and succinate, had no effect on growth, but others such as citrate, acetate, and malate totally inhibited growth; lactate slightly inhibited growth. The concentrations of alpha-ketoglutaric acid, pyruvate, and casein hydrolysate could be varied somewhat without affecting growth. Nevertheless, during several experiments in which growth was marginal because of limited casein hydrolysate or, in a medium which by itself did not support growth, 0.1% pyruvate stimulated growth. In experiments with the chemically defined medium, very small but definite increases in growth were observed when at least 0.1% of pyruvate was added.

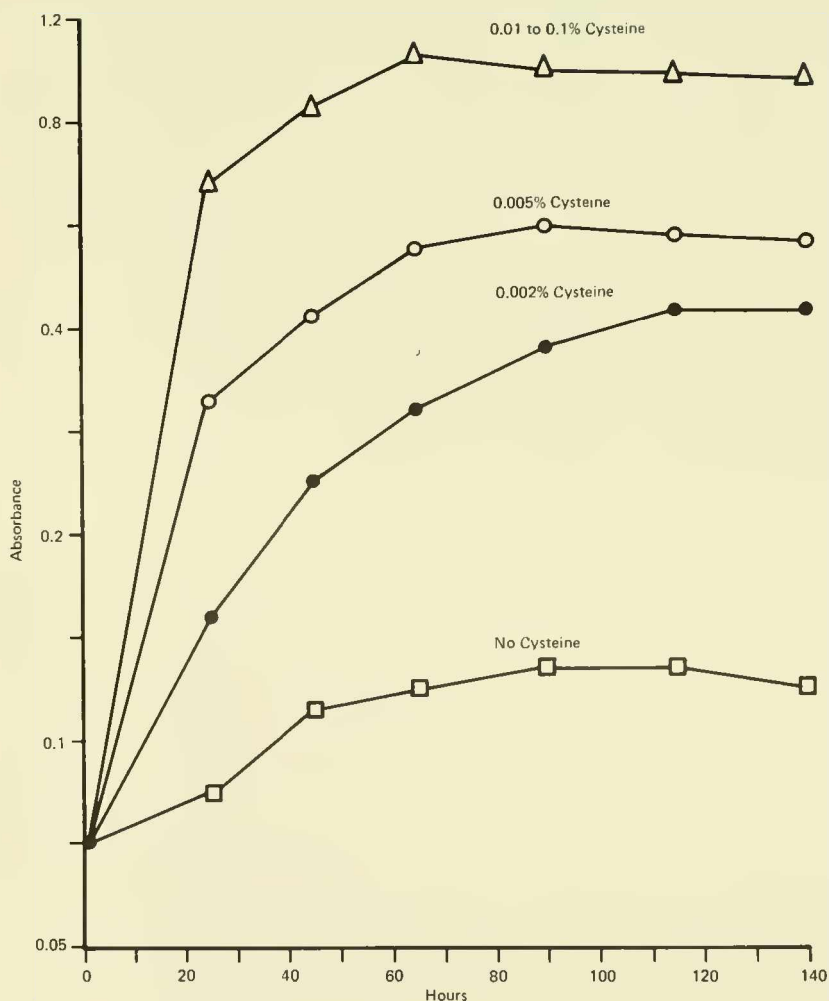
The results with glucose, polysaccharides, and organic acids show that the bacterium does not use these products as catabolic substrates for energy and carbon. The effects of alpha-ketoglutaric acid and pyruvate are complex, although there is little question that they are beneficial to growth. However, there is little evidence that they provide energy. It remains to be determined whether they are involved in buffering, transamination reactions, or are intermediaries or regulators of some metabolic process. When the media were being prepared, we noted that both alpha-ketoglutaric acid and pyruvate function as chelators because precipitates that formed as the pH was adjusted were reduced or eliminated.

### C. Nutritional requirements

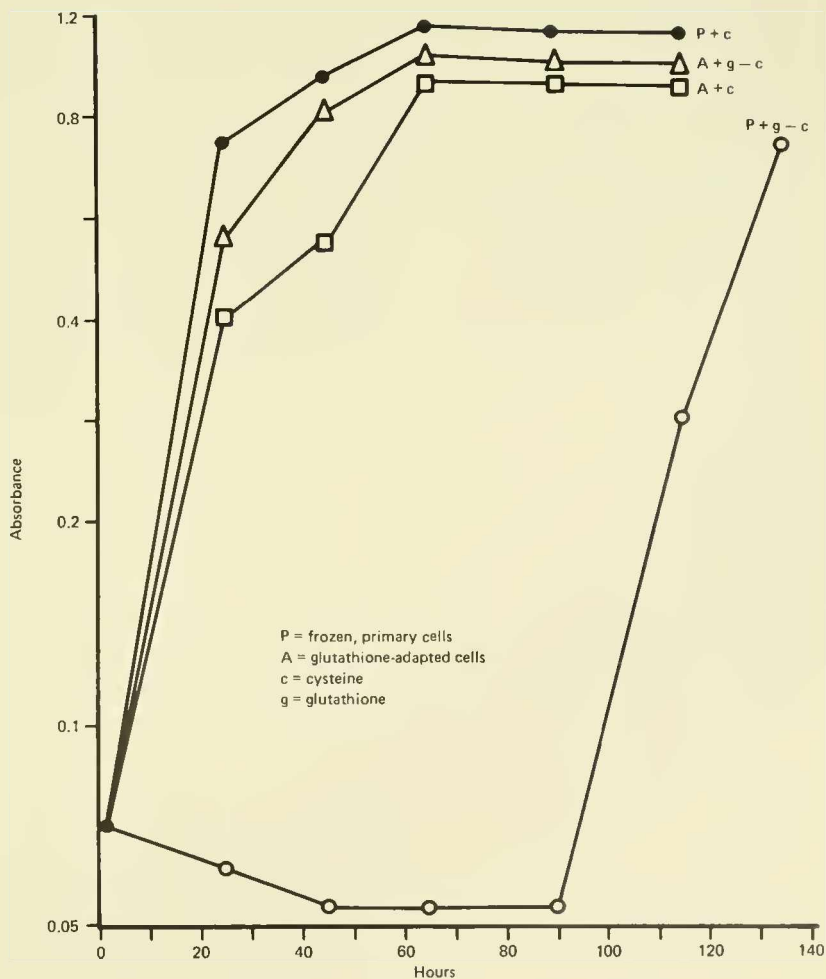
Cysteine was required for growth as previously reported (10), and growth was directly proportional to cysteine concentrations between 0.002% and 0.01% (Fig. 5); higher concentrations did not increase cell yields and were slightly inhibitory. Cystine did not substitute for cysteine, but glutathione did substitute for cysteine on an equimolar basis. Although the initial growth was delayed with unadapted cells, cells adapted to utilizing glutathione had equal growth rates and cell yields (Fig. 6). Under favorable growth conditions, the presence of 0.12 M dithiothreitol, 2-mercaptoethanol, thioglycolate, or methionine moderately to completely inhibited growth in medium containing only 0.02% cysteine. These and other results suggest that cysteine functions as a required amino acid and is not required as a reducing agent or chelator. Independent studies by one of us (JRG) showed that serine, methionine, arginine, valine, leucine, isoleucine, and threonine are also required for growth of at least 10 LDB strains representing four serogroups.

When all the vitamins (Part 1, steps 3-5, Table 1), starch, and oleic acid were removed, growth was delayed. However, all further attempts with several strains to demonstrate a vitamin requirement have failed, although one of us (MWR) used these same media to demonstrate thiamine and biotin requirements of *Escherichia coli*. Similarly, others have been unable to demonstrate any vitamin requirements in liquid synthetic media (W. J. Warren and R. D. Miller, personal communication).

In addition to the -SH compounds above which inhibited growth, 1  $\mu$ g/ml of oleic acid, in the absence of starch, completely inhibited growth; in the presence of 0.05% starch, 10  $\mu$ g/ml of oleic acid did not inhibit growth. Whether the fatty acids of agars are inhibitors is not yet known; nevertheless, it is known that 1% starch was optimal for agar media. The presence of 0.05% ether-purified Tween 80 instead of starch totally inhibited growth, as did ethylenediaminetetraacetic acid. Inhibition by the latter compound plus the observations of inhibition by citrate, acetate, and malate suggested that trace elements played a major role in metabolism. One of us (MWR) demonstrated a requirement for iron in the defined medium with alpha-alpha dipyridyl; other results indicate requirements for calcium and magnesium.



**Figure 5.** Requirement of the Legionnaires' disease bacterium for cysteine. The basal medium was the SSLp medium with no glutathione; CO<sub>2</sub> seals were used on tubes incubated at 37°C and shaken at 70 rpm.



**Figure 6.** Glutathione as a substitute for cysteine; growth of strain Philadelphia 1 of the Legionnaires' disease bacterium as frozen, primary cells or glutathione-adapted cells in the semisynthetic broth with 0.1% cysteine or with 0.254% glutathione and no cysteine.



The chemically defined broth was compared to the semisynthetic broth for maintaining growth in five sequential transfers of 10 strains of the LDB: Philadelphia 1, Philadelphia 2, Philadelphia 3, Philadelphia 4, Pontiac, Flint 1, Bellingham, Miami Beach, Detroit 1, and Togus 1. The defined broth (with no starch) supported growth of the tested strains equally well. Indeed, in one or more passages, the cell yields were higher than those observed in the casein hydrolysate broth—suggesting that some amino acids of the defined mixture were at levels preferable to those in the casein hydrolysate broth. The various strains of the organism generally adapted readily to the two media. Although results obtained with the different strains varied to some degree, in general we found that the defined medium containing 0.05% starch was superior to the casein hydrolysate medium.

#### D. Physiology

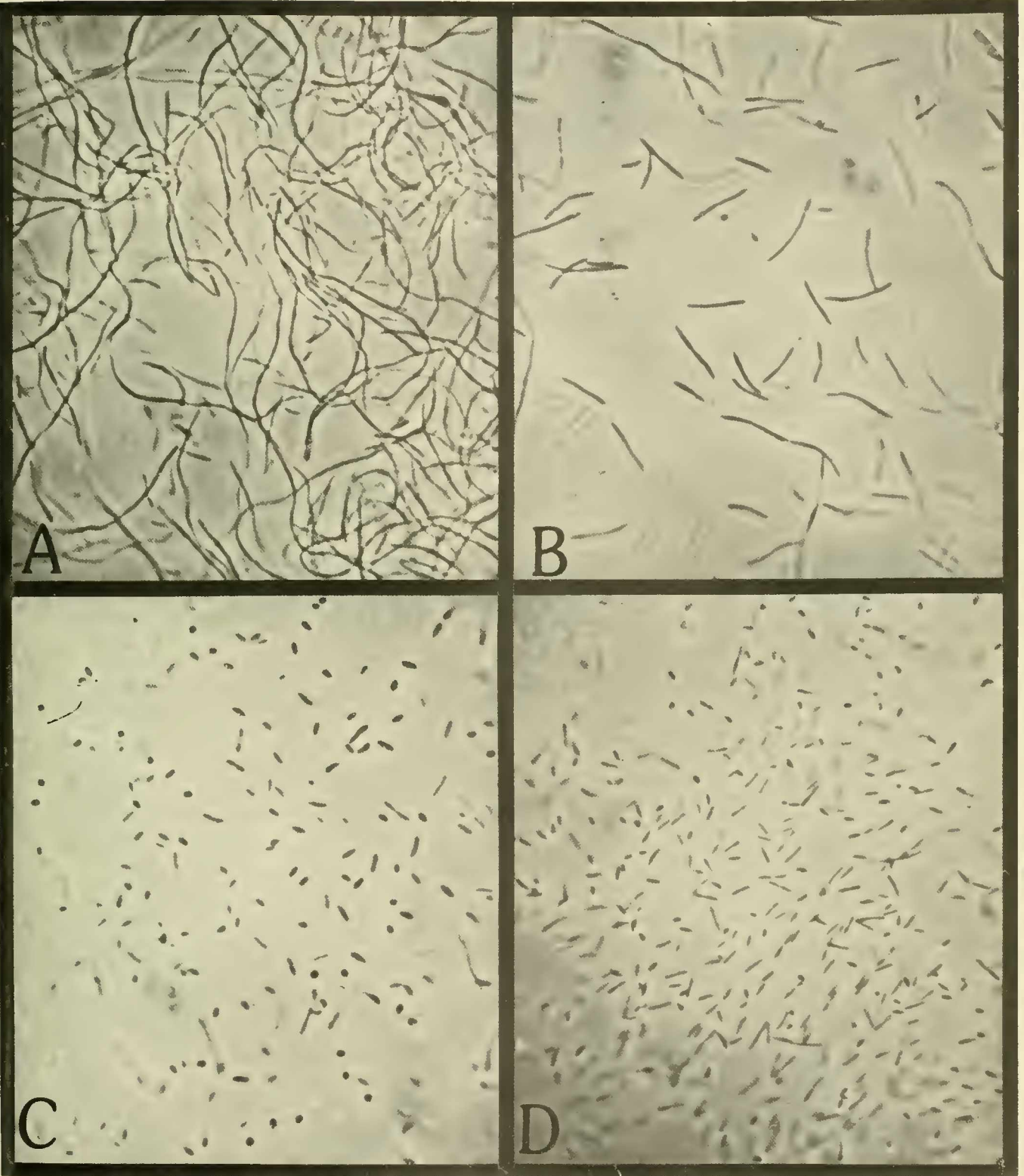
In most of the experiments with the various solid and liquid media, cells were examined for morphology, catalase production, and hydrolysis of starch. Cells were also stained with the serogroup-specific fluorescent antibody (FA) rabbit-anti LDB conjugate, and the presence of surface antigen was compared with that of cells grown on the F-G medium. Catalase production was extremely limited or absent, and no hydrolysis of starch was observed using any of these media. In all cases, cells grown on either the semisynthetic or chemically defined medium stained by direct FA as well as or better than control cells grown on F-G medium.

Morphology of the LDB varies widely under different growth conditions (Fig. 7). In general, growth covers the entire surface of an agar plate because of the liquid present; growth apparently starts sooner and is more rapid in high humidity. However, we did not observe spreading or crawling motility. When plates were incubated between NaOH pellets (to absorb  $\text{CO}_2$ ), the pellets also functioned as dessicants, and the surfaces were dried—producing discrete colonies with entire edges; results were similar when plates were incubated 7 to 10 days, and the colonies were typical of those grown on CYE medium. The slower growth observed under these conditions may have been the result of depleted  $\text{CO}_2$  or a dried surface or both. Cellular morphology changed progressively in broth, with large masses of filaments or chains of bacilli in the logarithmic phase breaking into shorter filaments and ultimately forming single and double cigar-shaped cells. With prolonged incubation or limited substrate, cells became coccoid shaped (Fig. 7). The organism grew at 25°C, 30°C, and 37°C. The cellular morphology was the same at 30°C and 37°C, but at 25°C cells appeared as fine, small bacilli (Fig. 7). We did not observe refractile cells or cells which might suggest spores or microcysts but did see swollen cell terminals which were probably spheroplasts.

#### SUMMARY AND DISCUSSION

In reviewing the characteristics of the LDB, we see a set of apparently conflicting growth requirements and physiological responses, which, according to the limited data presently available, may be more apparent than real. We have an aerobic organism which sometimes seems sensitive to excesses of oxygen, which can grow under the microaerophilic condition of the candle jar, which produces limited amounts of—if any—catalase, and which has a specific requirement of cysteine as an -SH amino acid. With the lability and extreme reactivity of the -SH of cysteine, particularly in alkaline solutions and in the presence of metals, it would not be difficult to visualize this as a factor limiting growth in nature or in laboratory media. Thus the limited aerobic requirements are supportive and not in conflict with this cysteine requirement. Furthermore, a preference for acid pH further supports a stable -SH form of cysteine because the molecule is highly reactive in alkaline conditions and is rapidly oxidized to cystine in the presence of iron. At neutral or alkaline pHs, cysteine spontaneously forms addition compounds with aldehydes, ketones, and unsaturated molecules (5, 7, 8).





**Figure 7.** Morphological aspects (1350 X) of the Philadelphia 1 strain of the Legionnaires' disease bacterium grown for:

- A. 3 days at 37°C in 0.8% casein hydrolysate semisynthetic broth with shaking in air.
- B. 4 days at 37°C in 0.8% casein hydrolysate semisynthetic broth with shaking in air + 5% CO<sub>2</sub>.
- C. 4 days at 37°C in 0.4% casein hydrolysate semisynthetic broth with shaking in air + 5% CO<sub>2</sub>.
- D. 7 days at 25°C on the semisynthetic agar slants (0.8% casein hydrolysate); incubated in air.

Many of these complexes are reversible, e.g., the reactions with glucose and pyruvate (8), but others may cause new stable compounds to form (7). Since aldehydes and keto compounds are less reactive under acid conditions, the marked stimulation by pyruvate and alpha-ketoglutaric acid at pH 6.5 compared to 7.2 may be due to lower reactivity of the acids with cysteine at pH 6.5. But the lower pH may also permit these acids to enter the cell more readily by shifting the dissociation equilibrium to the side of the non-ionized acids.

Although the LDB has shown sensitivity to citrate, malate, and acetate—recognized chelating agents of calcium, magnesium, and iron—pyruvate and alpha-ketoglutaric acid did not inhibit growth even though their chelating effects were observed when certain experimental media were being neutralized.

In addition, the organism must attempt to utilize amino acids as a source of energy and carbon, with pHs subsequently rising as high as 8.2. These amino acid substrates appear to be primarily serine and threonine. With the media we developed, the buffering effect of carbon dioxide is significant and can in part explain the results observed in liquid shake cultures where the CO<sub>2</sub> helped maintain an acid pH. However, carbon dioxide may have other functions and an acid pH would be contrary to its solution as bicarbonate.

Finally, the LDB appears to be quite sensitive to oleic acid; presumably this is the toxic factor in agar which is reversed by adding either 1.0% starch or 0.2% charcoal (Norit A). However, our experience with purified Tween 80 and with certain inocula suggest that the LDB itself may be a source of inhibiting fatty acids. Thus, with the excretion of a soluble lipase described by W. B. Baine et al. (Personal communication), one can visualize the release of toxic fatty acids from purified Tween 80 or from lysed cells of the inoculum.

Nevertheless, in the simple, chemically defined medium (Table 2) without added vitamins, oleic acid, and starch, 10 LDB strains tested have grown extremely well during several sequential transfers.

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# Electron Microscopy of the Legionnaires' Disease Bacterium

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## Electron Microscopy of the Legionnaires' Disease Bacterium

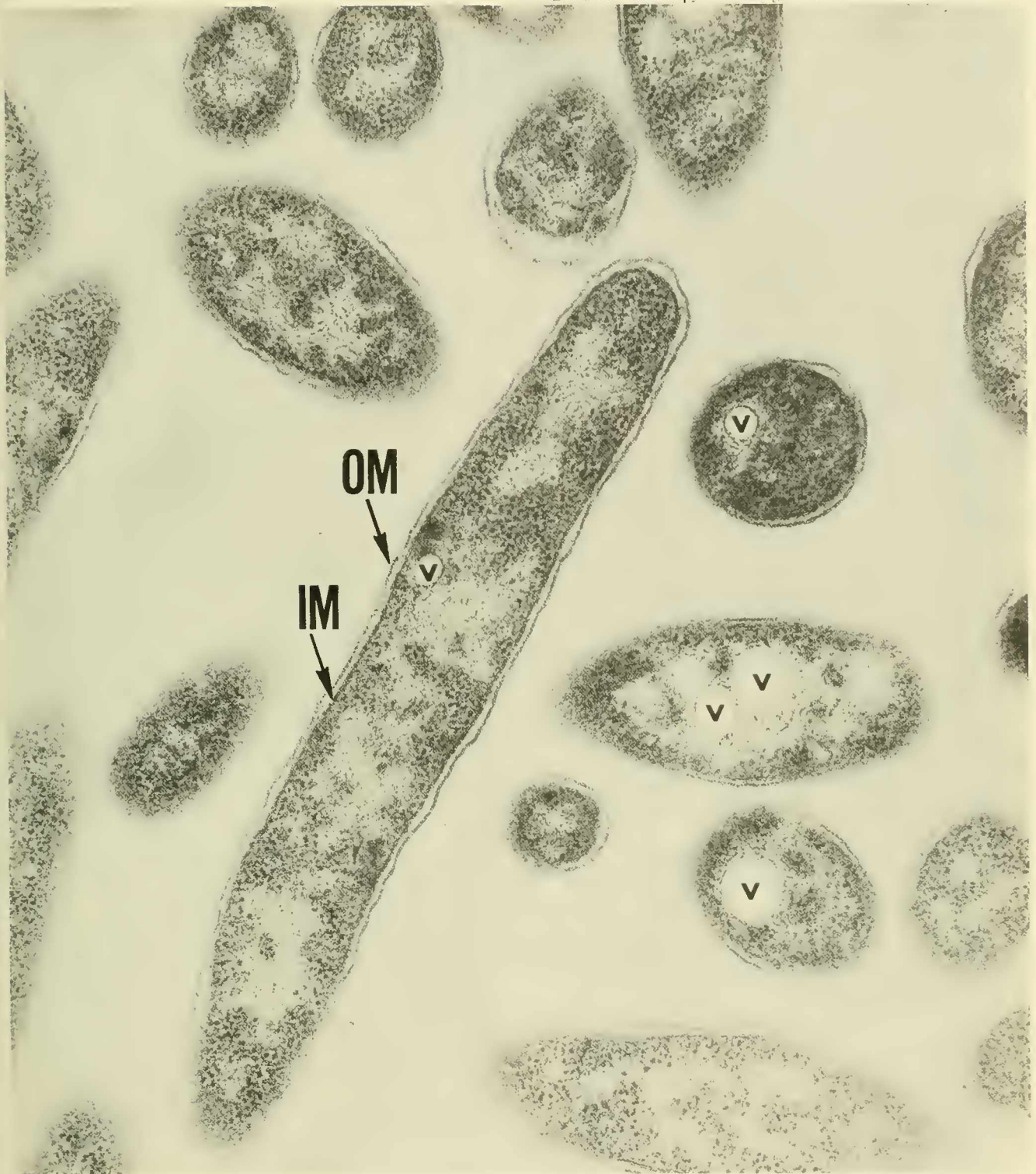
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We studied the Legionnaires' disease bacterium (LDB) by examining cultures, preparations of human lung tissue, and chicken egg yolk sacs with transmission electron microscopy (TEM). Samples of consolidated human lung were obtained at autopsy from twelve patients with confirmed Legionnaires' disease. Formalin-fixed pieces of these lung tissues were embedded in paraffin, sectioned  $\leq 5 \mu\text{m}$ , stained by the Dieterle silver impregnation method (2), and examined by light microscopy. If numerous bacteria characteristic of the LDB were seen, the piece of tissue from which the section was taken was diced into 1 mm cubes for TEM studies. Normal yolk sacs and those infected with the LDB were fixed either in cold 4% (v/v) buffered paraformaldehyde or cold 2% (v/v) glutaraldehyde in 0.2 M S-collidine buffer, pH 7.0, for 1 h. Both lung specimens and yolk sacs were postfixed in 1% osmium tetroxide (v/v), buffered in 0.2 M S-collidine, pH 7.2 to 7.4 (7), for 1 h at 4°C. The tissue specimens were then dehydrated through graded alcohols, embedded in Maraglas 732, and cut on a Reichert OMU2 ultramicrotome fitted with a diamond knife. Sections were picked up on uncoated copper grids and stained with saturated uranyl acetate and lead citrate (13). Selected 4-day cultures of the LDB in Mueller-Hinton broth supplemented with IsoVitaleX were prepared by the method of Ryter and Kellenberger (6). For whole cell preparations, broth cultures of the LDB were dried on Formvar-coated grids and stained with 2% aqueous uranyl acetate. All sections were examined with a Philips 200 transmission electron microscope at 40 KV.

The LDB is ultrastructurally identical in human lung tissue, experimental lesions of guinea pigs, bacteriologic media, or yolk sac membranes of embryonated hens' eggs. In sections, the LDB is a blunt or tapering rod which usually measures 0.3 to 0.9  $\mu\text{m}$  in diameter and  $\geq 2.0 \mu\text{m}$  in length (Figs. 1 and 2). Its sides may not be parallel, and it varies considerably in length depending on its environment. After 4 to 7 days of growth, greatly elongated forms are commonly found in cultures and less commonly found in yolk sac membranes; they are rarely found in human lung or in experimentally induced lesions in guinea pigs. The LDB is clearly prokaryotic (8,9) in that it lacks eukaryotic features such as mitochondria, nuclear membranes, endoplasmic reticulum, and mitotic division. Prominent features include electron-lucent, filamentous nucleoids interspersed among areas of well-defined ribosomes; enclosure by a double envelope, each portion of which consists of a triple-layered "unit" membrane (4,10); and division by a pinching, nonseptate process (Figs. 2 and 3). This pinching type of division and double envelope enclosure are characteristic of gram-negative bacteria (4, 5, 10, 11).

With the techniques used, we have not been able to see any definite structure that could represent a peptidoglycan layer (11) in the periplasmic space. Cleanly circumscribed, round electron-lucent vacuoles are frequently seen within the LDB (Fig. 1). These vacuoles stain readily with Sudan black B in smears made from cultures, and their lipid content is probably removed by the solvents used in preparing samples for electron microscopy. Their ultrastructural appearance is suggestive of poly- $\beta$ -hydroxybutyrate granules in sections (3,12). Rarely, membranous profiles





**Figure 1.** Electron micrograph of Legionnaires' disease bacteria grown on bacteriologic medium. Note enclosure of organisms by a double envelope consisting of inner (IM) and outer (OM) triple-layered "unit" membranes. Vacuoles (v) are present in some organisms. (osmium fixation, X90,000).

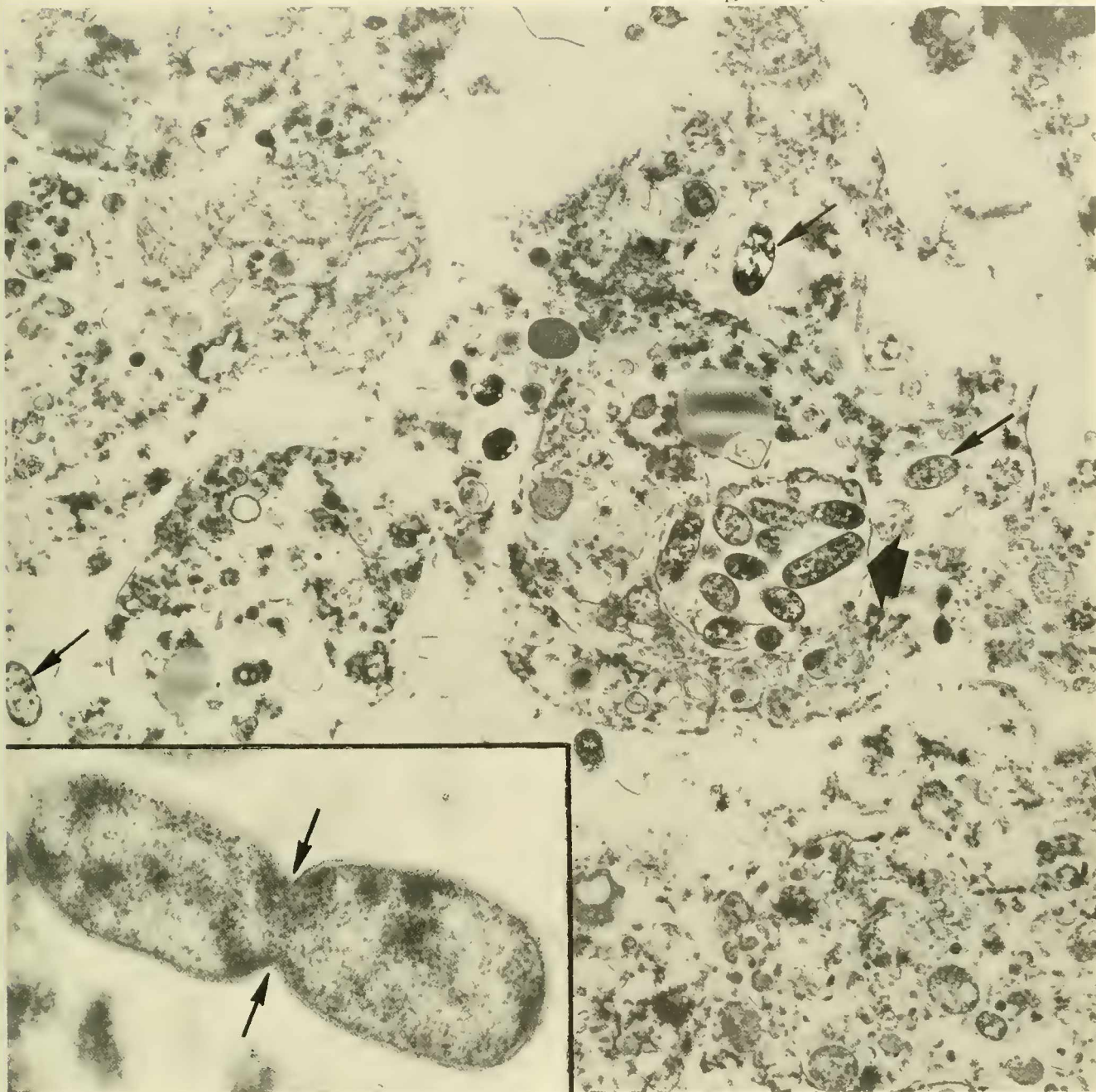


**Figure 2.** Whole preparation of Legionnaires' disease bacteria grown on bacteriologic medium. Note dividing organism (arrows indicate region of central pinching) and delineation of outer envelope (blunt arrows) (uranyl acetate, X43,700).

which appear to be extensions from the inner envelope are seen in the LDB cytoplasm. However, membranous inclusions or other organelles, such as those seen in some gram-positive bacteria (9), are not found in the LDB.

Autopsy specimens from patients with LD included lung tissues obtained during the 1976 Philadelphia outbreak as well as those from patients associated with sporadic or epidemic cases of LD since then. A confirmed diagnosis of LD was based either on results of indirect immunofluorescent serodiagnosis, direct immunofluorescence of lung sections or smears, isolation on artificial media, guinea pig inoculation, or some combination of the four as described elsewhere in this manual. The electron microscopic features of the LDB in areas of typical pulmonary consolidation, determined by light microscopy of hematoxylin-eosin and Dieterle silver impregnation stained sections (1,2), are consistent. However, the concentration of organisms varies from specimen to specimen and case to case. The LDB is predominately found in phagosomes of degenerating or necrotic alveolar macrophages (Fig. 3). Enormous phagosomes, which occupy most of the host cell cytoplasm, may contain as many as 40 organisms in a single plane of section. Extracellular LD bacteria may also be present and are often intimately associated with cellular





**Figure 3.** Electron micrograph of Legionnaires' disease bacteria within phagocytic vacuole (blunt arrow) of degenerating alveolar inflammatory cell. Extracellular organisms are also seen (arrows) (X12,750). **Inset:** Detail of dividing organism within alveolar macrophage. Note central pinching (arrows) and enclosure by a double envelope (uranyl acetate and lead citrate, X61,712).

debris and fibrin (Fig. 3). Multiplying as well as degenerating organisms are observed intracellularly and extracellularly. We have seen fewer LD bacteria within neutrophils and, when present, only one to six organisms usually occupy a phagocytic vacuole in one plane of section.

Because the ultrastructural features of the LDB are not unique, it may be impossible to differentiate this organism from other small gram-negative bacilli by fine structure alone. However, our studies confirm the characteristic gram-negative ultrastructure of the LDB and earlier reports of its gram-negative staining when isolated from cultures.

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# Cellular Fatty Acid Composition of the Legionnaires' Disease Bacterium

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## Cellular Fatty Acid Composition of the Legionnaires' Disease Bacterium

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Identification and classification of microorganisms has been a central theme in the development of microbiology as a science. Since the early work of Pasteur, classification has been based largely on morphological, physiological, serological, and biochemical data. In recent years, a new dimension—the use of chemical data—has been added to bacterial classification, and the term “chemotaxonomy” is now well-established in the literature. To the microbiologist, the term usually denotes techniques such as cell wall analyses, DNA composition, and DNA homologies. In a broader sense, the term includes studies of cell composition with respect to sugars, proteins, amino acids, and lipids, and studies of metabolic products of microorganisms.

For the last several years our laboratory has done chemotaxonomic studies using gas liquid chromatography, mass spectrometry, and associated analytical techniques to develop new methodology for rapid and sensitive detection, identification, and classification of organisms. We have done extensive studies with cellular fatty acids and have found that a number of closely related bacterial species can be distinguished on the basis of qualitative differences in their fatty acids (8). Moreover, we have observed that the cellular fatty acid compositions of strains of a species are essentially identical. Our initial interest in the Legionnaires' disease bacterium (LDB) was to examine the cellular fatty acid composition of all isolates to assess their degree of chemical relatedness. At the time the study was begun, few data were available from conventional cultural and biochemical tests. Thus, the data could provide valuable information for establishing the relationship of isolates obtained in the future and those from the 1976 Legionnaires' disease outbreak.

Four isolates from Philadelphia and an isolate each from Flint and Pontiac, Michigan, were included in the initial study. Each of the six strains was inoculated onto a plate of Mueller-Hinton agar supplemented with 1% hemoglobin and 1% (v/v) IsoVitaleX (BBL) and incubated in a candle jar at 35°C for 72 h. After about 3 ml of sterile distilled water was added, the heavy cell growth on the plates was removed with glass rod spreaders. Smears were prepared for Gram staining to check the purity of the cultures. The cells were saponified, and the fatty acids were methylated by the procedure described previously (10) and outlined in detail elsewhere in this manual. *Pseudomonas cepacia*, an organism of known cellular fatty acid composition (8), was used as a control; it was grown and processed under the same conditions as the six test cultures.

Methyl esters were analyzed on a Perkin-Elmer Model 900 gas chromatograph (Perkin-Elmer, Norwalk, CT) equipped with a flame ionization detector and a Disc integrator recorder. The instrument contained a 0.16-in (4.06 mm, I.D.) x 12-ft (3.66 m) coiled glass column packed with 3% OV-101 methyl silicone which was coated on 100-120 mesh Gas-Chrom Q (Applied Science Lab., State College, PA). The carrier gas was helium at a flow rate of 60 ml/min. The initial column temperature was 160°C; after the sample was injected, it was increased to 265°C at a rate of 5°C/min. Fatty acid methyl ester peaks were tentatively identified by comparing their retention times to those of methyl ester standards (Applied Science Lab.; Analabs, North Haven, CT; Supelco, Bellefonte, PA). Final identification was established by hydrogenation (10) and

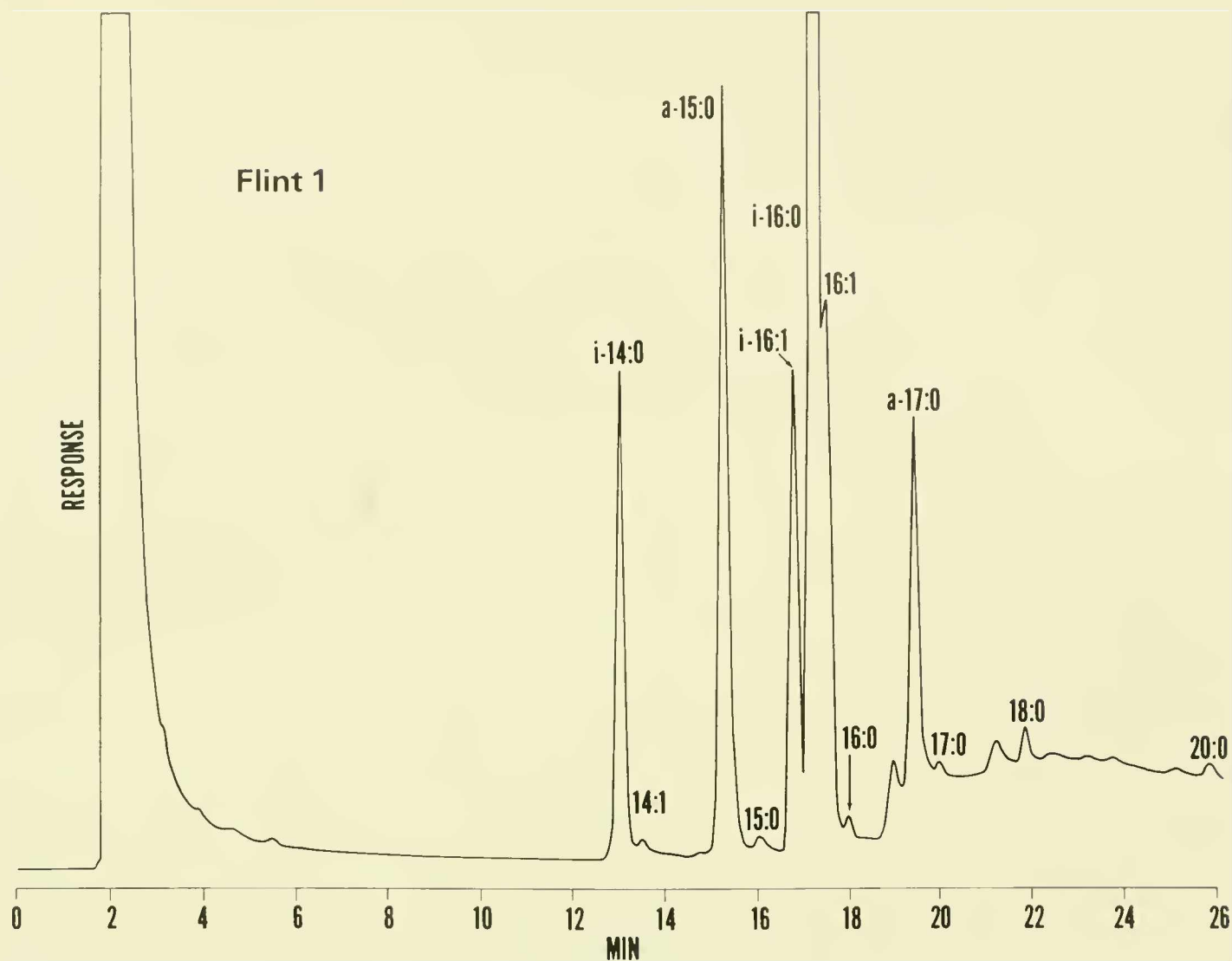


mass spectrometry (10, 16, 18). Combined gas chromatography-mass spectrometry of methyl esters was done with a DuPont instrument type 21-491B equipped with a combination electron impact(EI)-chemical ionization (CI) source. Isobutane was used as the reagent gas for CI. Details of the gas chromatographic procedure and identification of fatty acids are presented elsewhere in the manual.

The cellular fatty acid profile (as methyl esters) of the Flint 1 strain of the LDB is shown in the chromatogram in Fig. 1. The single most abundant acid in the chromatogram is a saturated branched-chain 16 carbon acid (i-16:0) with the methyl branch at the iso-(penultimate)carbon atom. The next most abundant are a mono-unsaturated 16 carbon straight chain acid (16:1), a saturated 15 carbon branched-chain acid (a-15:0) with the methyl branch at the anteiso-(anti-penultimate)carbon atom, a saturated 14 carbon branched-chain acid (i-14:0), a saturated 17 carbon branched-chain acid (a-17:0), and a mono-unsaturated 16 carbon iso-branched-chain acid (i-16:1). With the exception of 16:1, the normal straight-chain saturated (15:0, 16:0, etc.) and unsaturated (14:1) acids were present in only small to trace amounts. The identities of the labeled fatty acid methyl esters in the chromatogram were confirmed by both EI and CI mass spectrometry. The EI spectra of anteiso-branched methyl esters were clearly distinguished from iso-branched and normal straight-chain esters by comparing the ratio of the  $m/e = M-29$  and  $m/e = M-31$  peaks in the spectra. With anteiso esters the  $M-29$  is equal to or greater than the  $M-31$ , whereas the  $M-31$  peak is approximately twice the size of the  $M-29$  peak in iso-branched and normal straight-chain esters (16, 18). Unsaturation was confirmed by hydrogenating the methyl ester sample (10), which resulted in the disappearance of the i-16:1 and 16:1 peaks with concomitant increases in the size of the i-16:0 and 16:0 peaks, respectively. The absence of hydroxy acids was confirmed by the fact that there was no change in retention time of any peak in the chromatogram when the methyl ester sample was treated with trifluoroacetic anhydride (10). The profiles of the other five strains were strikingly similar, as shown in Fig. 2. The fatty acid profile of the control culture, *P. cepacia* was essentially the same as that reported previously (10).

The cellular fatty acid composition of each of the six strains is shown in Table 1. Peak areas from gas-liquid chromatography (GLC) were determined with the Disc integrator, and the percentage content of each acid was calculated from the ratio of the area of its peak to the total area of all peaks. Relative response factors determined for each acid were used in the calculation. The data clearly show the similarity of the fatty acid compositions of the six strains. In each strain the iso-16:0 acid was the major component; concentrations ranged from 32% to 43% of the total acid content. With the exception of 16:1, the other four major acids in each strain were also branched-chain acids. These acids (i-14:0, a-15:0, i-16:1, a-17:0) were present in each strain but in different proportions. The total proportion of branched-chain acids in each of the six strains ranged from 81% to 90%. The only qualitative difference in the cellular fatty acid profiles of the six strains was the presence of relatively small amounts (2%-4%) of a 17-carbon cyclopropane acid (17 $\Delta$ ) in the Pontiac and the Philadelphia 2, 3, and 4 strains, which was not detected in the other two strains. Results of retesting the cellular fatty acid composition of each strain through the entire procedure (growth, saponification, extraction, GLC) were virtually identical (see Table 1).

Since the initial study, 62 additional strains of the LDB have been isolated from clinical materials and from environmental sources at diverse geographical locations. We examined each of these for cellular fatty acids after growing them on enriched Mueller-Hinton (M-H) agar or on other recently developed growth media [Feeley-Gorman (F-G) agar (1), charcoal yeast extract (CYE) agar (1), semisynthetic medium (14)]. The cellular fatty acid composition of each strain was similar to that observed in the initial study (12, Figs. 1 and 2, and Table 1). No major differences in cellular fatty acids were observed among strains of the four recognized serogroups (7).



**Figure 1.** Gas chromatogram of esterified fatty acids from saponified whole cells of the Flint 1 strain of the Legionnaires' disease bacterium. Analysis was made on a 3% OV-1 column. Peak designation: number before the colon refers to the number of carbon atoms; number to the right refers to the number of double bonds; (i-) indicates a branched-chain acid with the methyl group at the penultimate carbon atom; and (a-) indicates a branched-chain acid with the methyl group at the anti-penultimate carbon.



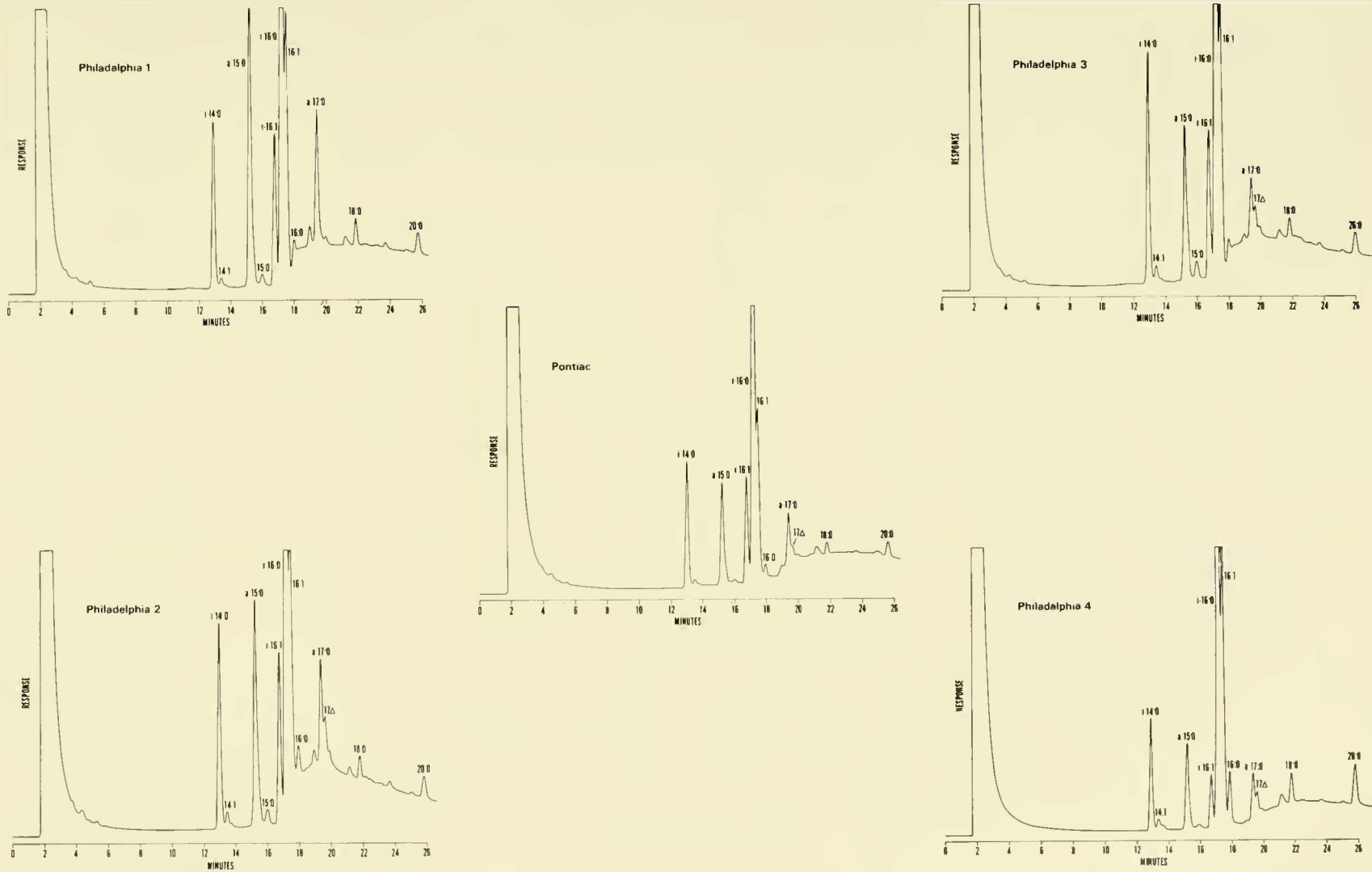


Figure 2. Cellular fatty acid profiles from gas liquid chromatography of 5 strains of the Legionnaires' disease bacterium.

Each new growth medium used for the LDB has been evaluated for its effect on cellular fatty acids. The fatty acid compositions of two LDB strains grown on M-H and CYE agars are described in Table 2. The data show no qualitative differences in fatty acids of cells harvested from the two media. However, the total proportion of branched-chain fatty acids was higher for cells grown on the M-H medium than for those grown on CYE agar (7% higher for strain Berkeley and 6% higher for strain Flint 2). The same relationship was observed for several other strains: the average total cellular branched-chain fatty acid content for 36 strains was 79% on M-H medium and 68% on CYE agar (Table 2). The cellular fatty acid compositions of strains grown on F-G agar and on Pine's semisynthetic medium were essentially identical to those of strains grown on

Table 1.  
Cellular fatty acid composition of six strains of the  
Legionnaires' disease bacterium.

Fatty Acid <sup>a</sup>	Philadelphia								Flint		Pontiac	
	1		2		3		4					
	#1	#2 <sup>b</sup>	#1	#2	#1	#2	#1	#2	#1	#2	#1	#2
i-14:0	11 <sup>c</sup>	12	13	11	14	14	10	13	11	11	11	12
14:1	T	T	T	T	T	T	T	T	T	T	T	T
14:0	T	T	T	T	T	T	T	T	T	T	T	T
a-15:0	20	20	13	14	14	11	12	13	20	20	11	12
15:0	T	T	T	T	T	T	T	T	T	T	T	T
i-16:1	11	11	11	11	13	9	12	10	12	12	11	10
i-16:0	34	32	39	42	35	43	40	40	36	32	40	40
16:1	13	14	14	15	16	14	15	14	10	14	14	14
16:0	T	T	T	T	T	T	T	T	T	T	T	T
a-17:0	11	11	8	5	5	5	7	6	11	11	9	8
17 Δ	-	-	2	2	3	4	4	4	-	-	4	4
17:0	T	T	T	T	T	T	T	T	T	T	T	T
Br 18:0	T	T	T	T	T	T	T	T	T	T	T	T
18:0	T	T	T	T	T	T	T	T	T	T	T	T
Br 19:0	T	T	T	T	T	T	T	T	T	T	T	T
19:0	T	T	T	T	T	T	T	T	T	T	T	T
Br 20:0	T	T	T	T	T	T	T	T	T	T	T	T
20:0	T	T	T	T	T	T	T	T	T	T	T	T
Total of branched-chain acids	87	86	84	83	81	82	81	82	90	86	82	82

<sup>a</sup> Number to the left of the colon refers to the number of carbon atoms; number to the right refers to the number of double bonds; i- indicates a methyl branch at the iso-carbon atom; a- indicates a methyl branch at the anteiso-carbon; Br indicates a branched-chain acid.

<sup>b</sup> Data obtained from a second experiment of cells processed through the entire procedure of growth, saponification, extraction, and GLC analysis.

<sup>c</sup> Numbers refer to percentages of total acids; T = less than 2%.

M-H agar, with some slight increase (5%-7%) in the relative amount of a-15:0 acid on the semisynthetic medium. Even though the relative proportions of total branched-chain fatty acids were lower for all strains grown on CYE agar than for those grown on M-H medium, LDB strains grown on any medium contained relatively large amounts of i-16:0, a-15:0, a-17:0, and i-14:0 acids.

In general, branched-chain fatty acids are characteristic of gram-positive bacteria (5, 6, 17), but they are present in major amounts (> 50% of total) in relatively few genera such as *Bacillus* (3, 4), *Listeria* (15), *Propionibacterium* (11), *Micrococcus* (2), and *Staphylococcus* (2). Of the gram-negative organisms, only *Thermus aquaticus* and *Flavobacterium* are known to contain major amounts of these acids (9, 13). A striking feature of the cellular fatty acids of LDB is their high content of branched-chain acids (> 77%). Although branched-chain acids have been found in other bacteria, their presence and relative concentrations and the absence of other acids which are generally present in other bacteria produce a unique cellular fatty acid profile for the LDB. Data show that this unique cellular fatty acid composition is a relatively consistent characteristic of the numerous LDB strains tested. The cellular fatty acid profiles are typical for cells from each growth medium developed to date. Limited data on some other factors known to influence cellular fatty acid composition (3, 6) indicate that they do not significantly affect the general fatty acid profile. Thus, determination of cellular fatty acid content is valuable for rapid identification and classification of suspected isolates of the LDB.

Table 2.  
Comparison of the cellular fatty acid composition of strains of  
the Legionnaires' disease bacterium grown on two different media<sup>a</sup>

Fatty Acid <sup>b</sup>	Berkeley 1		Flint 2		Av. of 36 Strains	
	M-H	CYE	M-H	CYE	M-H	CYE
i-14:0	7 <sup>c</sup>	7	7	6	10	8
14:0	T	T	T	T	T	T
a-15:0	17	21	14	18	15	14
15:0	T	T	T	3	T	T
i-16:1	7	7	3	4	6	3
i-16:0	36	26	49	35	39	32
16:1	7	14	10	12	15	13
16:0	7	5	7	8	2	10
a-17:0	17	16	10	12	9	11
17Δ	T	T	T	T	T	3
17:0	T	T	T	T	T	2
18:0	2	2	T	2	2	2
19:0	T	T	T	T	T	T
20:0	T	2	T	T	2	2
Total of branched-chain acids	84	77	83	77	79	68

<sup>a</sup> M-H; enriched Mueller-Hinton agar

CYE; charcoal yeast extract agar

<sup>b</sup> The number to the left of the colon refers to the number of carbon atoms; the number to the right refers to the number of double bonds; (i-) indicates a methyl branch at the iso-carbon atom; (a-) indicates a methyl branch at the anteiso-carbon; and (Δ) indicates a cyclopropane ring.

<sup>c</sup> Number refers to percentage (%) of total fatty acids; T = less than 2%.



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# Antimicrobial Susceptibility of the Legionnaires' Disease Bacterium

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## Antimicrobial Susceptibility of the Legionnaires' Disease Bacterium

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In 1976 an outbreak of acute respiratory disease, later called "Legionnaires' disease," occurred among people who had attended a convention in Philadelphia, Pennsylvania (5). The causative agent, later identified as a bacterium (9), is referred to as the Legionnaires' disease bacterium (LDB). Since 1976, more than 500 cases with 79 fatalities have been identified from outbreaks (2). As of October 31, 1978, 453 sporadic cases with 86 fatalities had been confirmed in 43 states and the District of Columbia (4).

A review of the antimicrobial therapy used for patients with Legionnaires' disease showed that case-fatality rates were highest for those treated with cephalosporins and were intermediate for those treated with aminoglycosides, chloramphenicol, ampicillin, or penicillin. Lowest rates were for patients who received tetracycline or erythromycin (5). Some patients have been successfully treated with a combination of intravenous oxytetracycline and rifampin (10). Doxycycline was effective in another patient, but gentamicin was not (7).

In vivo studies have been performed with a guinea pig animal model (3, 6, 11) and with an embryonated hens' egg model (8). In the first in vivo study of therapy for an infection in an animal model, erythromycin was demonstrated to be effective in treating infected guinea pigs (3). All of the guinea pigs treated with erythromycin survived, whereas all the infected controls died. Later the same investigators used five other antimicrobial agents to treat guinea pigs infected with lethal doses of the LDB (11). The most effective drug was minocycline hydrochloride, which was associated with a 50% survival rate. Rifampin was less effective than minocycline, and the aminoglycosides (gentamicin, tobramycin, and amikacin) were essentially ineffective, even though they are very active in vitro.

In another in vivo study, guinea pigs were infected with the LDB and then treated with antibiotics (6). Erythromycin and rifampin were the most effective drugs in preventing mortality. Penicillin, chloramphenicol, tetracycline, and gentamicin had no significant effect (6).

In the embryonated hens' egg model studies (8), rifampin (followed by gentamicin, streptomycin, erythromycin, sulfadiazine, and chloramphenicol, in that order) most effectively prevented death of the embryo. The least effective drugs were cephalothin, which protected only if large amount were used, and oxytetracycline, which partially protected embryos. Ampicillin at the highest levels tested did not prevent or delay death of the embryo (8).

In vitro studies, with agar dilution and broth microdilution techniques, showed that rifampin was the most active agent, but that the macrolides (erythromycin and roxithromycin), the aminoglycosides (gentamicin, tobramycin, and amikacin), cotrimoxazole (sulfamethoxazole, 19 parts plus trimethoprim, 1 part), and chloramphenicol were very active (12, 13). Minimum inhibitory concentrations (MICs) obtained in these studies indicate that the LDB would be only slightly susceptible to commonly used  $\beta$ -lactam antibiotics (cephalosporins and penicillins). The most active  $\beta$ -lactam antibiotic tested was cefoxitin (a cefamycin, which has only recently been approved by the Food and Drug Administration for general use) (12).



All clinical and environmental strains of the LDB tested produce a  $\beta$ -lactamase which is more active on cephalosporins than on penicillins (13). The greater activity of cefoxitin on these organisms probably reflects its resistance to the  $\beta$ -lactamase produced by the LDB.

On the other hand, cefamandole, a cephalosporin that is resistant to some  $\beta$ -lactamases of gram-negative bacilli, is not resistant to the LDB  $\beta$ -lactamase. As new  $\beta$ -lactam antibiotics are synthesized, they should be tested for susceptibility to the enzyme of the LDB. If they are resistant, they should be candidates for clinical trials of treatment for Legionnaires' disease.

The in vitro results show that minocycline and doxycycline are more active than tetracycline. All the drugs (except for cotrimoxazole) that were active on the LDB on the basis of MICs were bactericidal at levels comparable to the MIC. The minimum bactericidal concentrations (MBCs) for cotrimoxazole were 33-fold greater than the MIC, but were still within achievable blood levels.

In vitro susceptibility results must be interpreted cautiously because of the difficulty in growing the organisms. None of the artificial media supports the rapid growth that is needed for susceptibility tests.

One reason for the lack of correlation between the in vitro and in vivo results may be the relative differences in the intracellular bactericidal action of the drugs. The LDB are frequently found within macrophages in lung specimens from patients who died with Legionnaires' disease (1) and within peritoneal macrophages in experimentally infected guinea pigs (Chandler, F., cited in 6). This may explain why rifampin and erythromycin are effective both in vitro and in vivo, whereas an aminoglycoside is effective only in vitro.

All strains of LDB that have been tested have shown similar susceptibility patterns. Because of this, and because the methods for performing susceptibility tests are far from optimal, we recommend that susceptibility tests not be performed routinely. Reference laboratories, such as ours, can monitor future isolates for changes in susceptibility.

All the LDB have produced a  $\beta$ -lactamase when tested by a chromogenic cephalosporin test (see Appendix) but not by the starch-iodine or acidometric techniques (13). The chromogenic cephalosporin test is very simple to perform. Although  $\beta$ -lactamase data may not be useful in formulating therapeutic strategy, they may be useful in characterizing the LDB.

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# *Legionella pneumophila* sp. nov.: The Legionnaires' Disease Bacterium

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## *Legionella pneumophila* sp. nov.: The Legionnaires' Disease Bacterium

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In the 2 years since the outbreak in Philadelphia (7) that brought Legionnaires' disease worldwide attention and led to the isolation of the Legionnaires' disease bacterium (LDB) at the Center for Disease Control (11), intense effort has been focused on understanding the "new" disease and the "new" bacterium. We have learned that the LDB is a gram-negative, weakly oxidase-positive, catalase-positive, fastidious organism that has narrow optimal pH and temperature ranges and will not grow anaerobically. It does not reduce nitrates, utilize carbohydrates, degrade urea, or appear to possess decarboxylases for lysine and ornithine or an arginine dihydrolase (19). It has a unique fatty acid profile and no major differences in cellular fatty acids among strains (15). Four different serogroups have been identified (6, 12).

Deoxyribonucleic acid (DNA) hybridization has proved extremely useful in the classification of bacteria (1, 8, 16, 17). We have characterized the DNA of LDB by genome size, guanine plus cytosine (GC) content, and DNA hybridization in order to determine the extent of relatedness between strains of LDB and possible relatedness of the LDB to other microorganisms.

DNA relatedness studies were first done before biochemical data were available for LDB and before its GC composition had been determined. Thus the initial experiments were designed to rule on the possibility that the LDB was a fastidious *Klebsiella* or another fastidious member of Enterobacteriaceae. Since DNA relatedness studies have previously been done on all described species within Enterobacteriaceae (1, 2, 3), we were able to test possible relatedness between the LDB and Enterobacteriaceae. DNAs from Philadelphia 1 and Flint 1 were reacted with labeled DNA from *Escherichia coli* K-12, *Proteus mirabilis* 1, *Yersinia enterocolitica* 498-70, and *Edwardsiella tarda* 3889-64 (Table 1). Relatedness was between 0% and 3% (5). All members of Enterobacteriaceae are at least 15% related to one or more of these four species (1, 2, 3). Therefore, the LDB is not a member of Enterobacteriaceae. Relatedness values below 5% are not considered to be significant. Similar experiments proved that LDB had no relatedness to *Staphylococcus epidermidis*, *Bordetella pertussis*, *Aeromonas hydrophila*, and *Vibrio cholerae* (Table 1). Although *Vibrio parahaemolyticus* and *Vibrio alginolyticus* were not tested directly (Table 1), these and some other vibrios were ruled out on the basis of their known DNA relatedness to *V. cholerae*.

Preliminary approximations, using optical thermal denaturation, indicated that the GC content of LDB DNA was slightly lower than that of *Proteus mirabilis*. GC content in strains of *P. mirabilis* is 39% to 42%. Quantitative determinations of GC content by optical thermal denaturation (9) and by CsCl buoyant density ultracentrifugation (10) showed that both Philadelphia 1 and Flint 1 DNA contained 39% GC. With this knowledge we were able to restrict DNA relatedness tests mainly to those organisms with known GC values between 35% and 43%.

Highest priority was given to determining relatedness between LDB and the organisms shown in Table 2. *Rochalimaea* (formerly *Rickettsia*) *quintana*, which has a GC content of 37%, was given high priority because its pattern of growth on artificial media is comparable to that of

the LDB. Since the LDB was initially isolated by methods designed for rickettsiae (11), comparisons with *R. quintana* were especially indicated. *Flavobacterium meningosepticum* and *Flavobacterium* IIB were tested because these organisms have similarities to the LDB. The only known group of gram-negative organisms that have predominantly branched-chain fatty acids are in the genus *Flavobacterium* (15). They are pigmented, their antibiotic resistance profile is similar to that of the LDB (18), and the GC content in the DNA of some flavobacteria is similar to that of the LDB. *Francisella tularensis*, *Yersinia*, and *Pasteurella multocida* were tested because some patients from whom *F. tularensis* and *Yersinia pestis* had been isolated had elevated serum titers to the LDB. Additional species in the genus *Pasteurella* and several species found in the environment were also tested.

Table 1.  
DNA relatedness of LDB strains to several species  
selected at random

Reaction <sup>a</sup>	% DNA relatedness at 60°C <sup>b</sup>
<i>E. coli</i> K-12*/ <i>E. coli</i> K-12	100 (85)
<i>E. coli</i> K-12*/Philadelphia 1	3
<i>E. coli</i> K-12*/Flint 1 (D8711)	1
<i>P. mirabilis</i> 1*/ <i>P. mirabilis</i> 1	100 (74)
<i>P. mirabilis</i> 1*/Philadelphia 1	2
<i>P. mirabilis</i> 1*/Flint 1 (D8711)	3
<i>Y. enterocolitica</i> 498-70*/ <i>Y. enterocolitica</i> 498-70	100 (74)
<i>Y. enterocolitica</i> 498-70*/Philadelphia 1	0
<i>Y. enterocolitica</i> 498-70*/Flint 1 (D8711)	0
<i>E. tarda</i> 3889-64*/ <i>E. tarda</i> 3889-64	100 (64)
<i>E. tarda</i> 3889-64*/Philadelphia 1	2
<i>E. tarda</i> 3889-64*/Flint 1 (D8711)	0
<i>V. cholerae</i> 1845-73*/ <i>V. cholerae</i> 1845-73	100 (81)
<i>V. cholerae</i> 1845-73*/ <i>V. cholerae</i> 1320-72	100
<i>V. cholerae</i> 1845-73*/ <i>V. cholerae</i> 5540-70	95
<i>V. cholerae</i> 1845-73*/ <i>V. parahaemolyticus</i> A8633	32
<i>V. cholerae</i> 1845-73*/ <i>V. alginolyticus</i> C6814	22
<i>V. cholerae</i> 1845-73*/Philadelphia 1	0
<i>V. cholerae</i> 1845-73*/Flint 1 (D8711)	0
<i>A. hydrophila</i> */ <i>A. hydrophila</i>	100 (67)
<i>A. hydrophila</i> */Philadelphia 1	0
<i>A. hydrophila</i> */Flint 1 (D8711)	0
<i>S. epidermidis</i> */ <i>S. epidermidis</i>	100 (61)
<i>S. epidermidis</i> */Philadelphia 1	1
<i>B. pertussis</i> */ <i>B. pertussis</i>	100 (65)
<i>B. pertussis</i> */Philadelphia 1	0
<i>B. pertussis</i> */Flint 1 (D8711)	0

<sup>a</sup> Labeled DNA is indicated by an asterisk.

<sup>b</sup> The figures in parentheses are the actual values obtained in homologous reactions. These are arbitrarily designated 100%, and heterologous values are normalized to them. The *B. pertussis* reactions were done at 65°C due to the high GC of *B. pertussis* DNA.



Table 2.  
DNA relatedness of LDB strain Philadelphia 1 to organisms  
of similar guanine plus cytosine content and organisms isolated  
from patients with high serum titers to LDB

Source of unlabeled DNA	% relatedness to LDB strain Philadelphia 1 <sup>a</sup>	
	60°C	75°C
<i>Rochalimaea quintana</i> ATCC VR358	5	0
<i>Flavobacterium meningosepticum</i>	2	
<i>Flavobacterium</i> IIB B8190	3	
<i>Flavobacterium</i> IIB B6829	3	
<i>Francisella tularensis</i>	4	1
<i>Yersinia enterocolitica</i> 498-70 <sup>b</sup>	0	
<i>Y. enterocolitica</i> 27 <sup>b</sup>	0	
<i>Y. enterocolitica</i> 1474 <sup>b</sup>	0	
<i>Y. enterocolitica</i> 48 <sup>b</sup>	0	
<i>Y. enterocolitica</i> 867 <sup>b</sup>	4	
<i>Y. pseudotuberculosis</i> P262	0	
<i>Y. pseudotuberculosis</i> P27	0	
<i>Pasteurella haemolytica</i> KC228	3	
<i>P. multocida</i> ATCC 12947	1	0
<i>P. multocida</i> ATCC 10544	1	0
<i>P. multocida</i> ATCC 6535	0	0
<i>P. ureae</i> KC518	7	4
<i>Cytophaga johnsonae</i> ATCC 29586	0	2
<i>Cytophaga johnsonae</i> ATCC 29588	0	0
<i>Flexibacter canadensis</i> UASM 9D	0	0
<i>Microcycus major</i> B859	3	0

a DNA reassociation in homologous Philadelphia 1 was approximately 70% in 60°C reactions and 60% in 75°C reactions.

b There are four different DNA relatedness groups among strains presently called *Yersinia enterocolitica* (3). These groups are characterized by their reactions for sucrose (suc), rhamnose (rha), raffinose (raf), and melibiose (mel). *Y. enterocolitica* strains 498-70 and 27 give the typical fermentation pattern: suc+, rha-, raf-, mel-. Strain 1474 is atypically suc-. Strain 48 is atypically rha+, raf+, mel+. Strain 867 is atypically rha+.

*Pasteurella ureae* and LDB were 7% related in 60°C reactions and 4% related in 75°C reactions. DNA relatedness of Philadelphia 1 to all these organisms at 60°C was 5% or less (Table 2). None of these values below 5% was considered significant. Therefore, the LDB is not related to any of these organisms at the species or genus level. While *Y. pestis* was not directly ruled out, it is more than 90% related to *Y. pseudotuberculosis* (13) and can be ruled out indirectly.

The time required for reassociation was similar for LDB DNA and DNA from *E. coli* K-12. DNA from this *E. coli* strain has a molecular weight of  $2.5 \times 10^9$  daltons. Therefore, LDB DNA is also approximately  $2.5 \times 10^9$  daltons; enough genetic information to specify some 3,000 genes. Isolates of LDB are extremely similar in growth, serology, biochemical tests, fatty acid composition, and antibiotic susceptibility profiles (11, 15, 18). DNA relatedness was determined for 15 of the LDB isolates available at the Center for Disease Control (CDC) to see if these relatively few phenotypic markers reflected similarity over the entire genome of LDB. Most



workers find that members of a given species are at least 70% related (1, 8, 16, 17). It has been our experience that relatedness among strains of a species remains above 60% when reactions are done at the stringent 75°C incubation temperature. Furthermore, divergence in DNA relatedness between strains of a species is almost always less than 6% (1, 2). Strains of LDB were 70% to 99% related to Philadelphia 1 (Table 3). Divergence in related sequences was 3% or less, and relatedness remained almost unchanged in 75°C reactions. Thus on the basis of these tests all of these LDB isolates belong to a single species.

Table 3.  
DNA relatedness among 15 strains of LDB

Source of unlabeled DNA	Reaction with labeled Philadelphia 1 DNA <sup>a</sup>		
	% relatedness		% divergence
	60°C	75°C	
Philadelphia 1	100(74)	100(60)	0(87.8°C)
Philadelphia 2	96		
Philadelphia 3	94	90	
Philadelphia 4	93	100	2.0
Pontiac 1	79	74	2.5
Flint 1 (D8711)	90	74	2.5
Flint 2 (E1035)	98		
Knoxville 1	84	80	3.0
Burlington 1	86	93	0.5
Bellingham 1	81	82	2.5
Albuquerque 1	94	77	3.0
Berkeley 1	97		
Bloomington 1	80	71	1.8
Bloomington 2	85	85	2.2
Togus 1	85		

<sup>a</sup> Figures in parentheses are the actual % of DNA reassociation in homologous Philadelphia 1 reactions, and the  $T_m$  (temperature at which 50% of labeled DNA is detected as single-stranded) for homologous, reassociated DNA. % divergence is the decrease in  $T_m$  (in °C) of heterologous DNA duplexes compared to that of the homologous DNA duplexes. It can be expressed as % because each degree decrease in thermal stability is caused by approximately 1% unpaired bases in double-stranded DNA (3).

The first four strains of LDB isolated were called Philadelphia 1, 2, 3, and 4. Strains subsequently isolated were also identified by city to distinguish them from the Philadelphia strains. This convention has now been adopted at CDC, and all strains are designated by city of origin and the number of the isolate from that city. For instance, Flint 1 and Flint 2 were both isolated in Flint, Michigan. If a strain has a city designation without a number, it can be assumed that this is the first isolate from that city: Albuquerque = Albuquerque 1. Specific culture collection numbers are put in parentheses after the city name and number: Flint 1 (D8711), Flint 2 (E1035), etc.

LDB strains Philadelphia 2, 3, and 4 are very closely related to Philadelphia 1. This is not surprising since these strains were all isolated from the same outbreak. The strain from the Burlington outbreak and strains from sporadic cases were also very closely related to Philadelphia 1.

LDB strains Togus 1, Bloomington 1, and Bloomington 2 were at least 80% related to Philadelphia 1 in reactions done at 60°C. These results are significant in that both the Togus and Bloomington isolates were serologically atypical and/or isolated from the environment. Togus 1 is the first strain whose O antigen does not react with the direct fluorescent antibody conjugate to LDB Knoxville (12). Bloomington 1 reacts with the Knoxville conjugate, but was isolated from the environment (14). Although Bloomington 2 is also an environmental isolate, it is serologically different from both Knoxville and Togus 1 (12, 14).

It is an impossible task to prove directly that LDB is different from all described species at the level of species, genus, and family. Our approach to this problem was to assume that LDB could not be related to organisms that had GC content in their DNA outside the range of 35% to 45%, or were gram-positive, strict anaerobes, spore formers, psychrophiles, thermophiles, acidophiles, or alkaliphiles. One can argue with any of these criteria, but the alternative is an irrational screening approach.

Genera containing one or more species still under consideration for some level of relatedness to LDB are listed in Table 4. Direct or indirect DNA relatedness results can be used to rule out at least most species in these genera except for those of *Acinetobacter*, *Bdellovibrio*, *Branhamella*, *Flexithrix*, *Kingella*, *Microscilla*, *Moraxella*, and *Simonsiella*. There are persuasive reasons to exclude these remaining genera. For example, *Branhamella* are cocci; *Flexithrix* are catalase negative, gelatin negative, and grow well at alkaline pH. One cannot, however, rule out the possibility that LDB is related to these genera at the family level. Therefore, species in all of these genera should be tested for DNA relatedness to LDB.

Table 4.  
Genera in which relatedness to *Legionella pneumophila* sp. nov.  
has not been totally ruled out

<i>Acinetobacter</i>	<i>Flavobacterium</i>	<i>Kingella</i>
<i>Actinobacillus</i>	<i>Francisella</i>	<i>Microscilla</i>
<i>Bdellovibrio</i>	<i>Flexibacter</i>	<i>Moraxella</i>
<i>Branhamella</i>	<i>Flexithrix</i>	<i>Pasteurella</i>
<i>Cytophaga</i>	<i>Haemophilus</i>	<i>Simonsiella</i>

It is unlikely that the LDB is related to any well-described pathogen. We believe that the LDB is a previously undescribed species. Its biochemical reactions, growth pattern, and DNA relatedness are sufficiently unique to warrant placing it in a new genus. The fact that to date LDB is apparently totally unrelated to other organisms is consistent with the creation of a new family.

We have proposed the name *Legionella pneumophila* McDade et al. (11) sp. nov. for the LDB. Le·gi·on·el·la. M.L. *legio* M.L.n. legion or army; M.L. dim. ending *ella*. pneu·moph·ila. Gr. n. *pneumo* lung; Gr. adj. *philos* loving (4). The type strain of *Legionella pneumophila* is Philadelphia 1. *Legionella pneumophila* was proposed as the type species of the genus *Legionella*. *Legionella* was proposed as the type genus for a new family Legionellaceae (4).

## SUMMARY

DNA from strains of the LDB was characterized in order to aid in the proper classification of this organism. The genome size of LDB DNA was estimated at  $2.5 \times 10^9$  daltons by reassociation kinetics; a GC content of 39% for LDB DNA was established by optical thermal denaturation and buoyant density ultracentrifugation measurements.



DNA relatedness was used to classify strains of the LDB. These DNA comparisons showed that all strains of LDB were members of the same species. Included were strains isolated from the environment and strains with three different O antigens. DNA from LDB was not significantly related to DNA from any other group of bacteria tested. Biochemical data, growth characteristics, and GC ratios were used to rule out the possibility that LDB was significantly related to members of genera whose DNA was not tested.

On the basis of these data, we propose that LDB be named *Legionella pneumophila* sp. nov., the type species of the genus *Legionella*.

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*Cytophaga johnsonae* UASM E-1-25 (ATCC 29586), *C. johnsonae* UASM 4539 (ATCC 29588), and *Flexibacter canadensis* UASM 9D were sent to CDC by P. Christensen of the University of Alberta Soil Microbiology Laboratory. They were cultivated and given to us by J. C. Feeley. *Pasteurella haemolytica* KC228 and *P. ureae* KC518 were cultivated and given to us by R. E. Weaver.

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# **PART THREE**

## **Methodology**





# Primary Isolation Using Guinea Pigs and Embryonated Eggs

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# Primary Isolation Using Guinea Pigs and Embryonated Eggs

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The properties of the Legionnaires' disease bacterium (LDB) have already been described. Briefly, the LDB is a gram-negative, non-acid-fast bacterium that does not grow on most bacteriologic media (7) but can be cultivated on enriched Mueller-Hinton (M-H) agar (9), F-G agar (3), a semi-synthetic medium derived from the supplemented M-H formula, and charcoal yeast extract (CYE) agar, recently developed for cultivating the bacterium (2).

The LDB was originally isolated in guinea pigs from autopsy specimens and propagated in embryonated hens' eggs (7). Later, after the organism was successfully cultivated *in vitro*, comparison studies were done to evaluate the relative sensitivities of the various isolation procedures (6). Although guinea pigs were found to be somewhat less sensitive than bacteriologic media for isolating the LDB, the fact that these animals are relatively resistant to most other bacteria enhances the likelihood of isolating the LDB from specimens that contain contaminants. Admittedly, the contamination problem with the *in vitro* systems could be solved by the addition of appropriate antibiotics (5, 12), but the fact remains that guinea pigs and embryonated eggs are good systems for isolating the LDB, having been used successfully in the first isolations of the LDB from clinical (7) and environmental samples (4, 11).

The following sections outline the procedures involved in isolating the LDB, with particular emphasis on using guinea pigs and embryonated eggs. Although the discussion is aimed specifically at isolating the LDB from human lung tissues, the procedures can be modified slightly and used for other types of specimens.

## COLLECTION AND PROCESSING OF SPECIMENS (see Figure 1)

Several thumbnail-sized pieces of lung tissue should be obtained from the affected areas at autopsy. At least one tissue sample should be placed in 10% neutral Formalin and used in subsequent pathology studies and in direct fluorescent antibody (FA) tests (1) for the presence of the LDB. Another piece of tissue should be stored at  $-70^{\circ}\text{C}$  as reference material, and the remaining tissue should then be taken or sent to the appropriate diagnostic laboratory for *in vitro* isolation attempts.

If these initial isolation efforts are unsuccessful, *in vivo* isolation can be attempted with guinea pigs and embryonated eggs if appropriate facilities are available. If not, tissue suspensions should be prepared and shipped to the appropriate reference laboratory in accordance with the instructions in Part Four of this manual. Specimens received by the reference laboratory can be safely held at  $-70^{\circ}\text{C}$  until processed.

### A. Initial Processing (see Figure 1)

(NOTE: All personnel who work with specimens suspected of containing the LDB should wear surgical masks and gloves, and specimens should be processed in a biological safety cabinet.) Grind a piece of lung tissue with a sterile mortar and pestle using alundum as an abrasive.

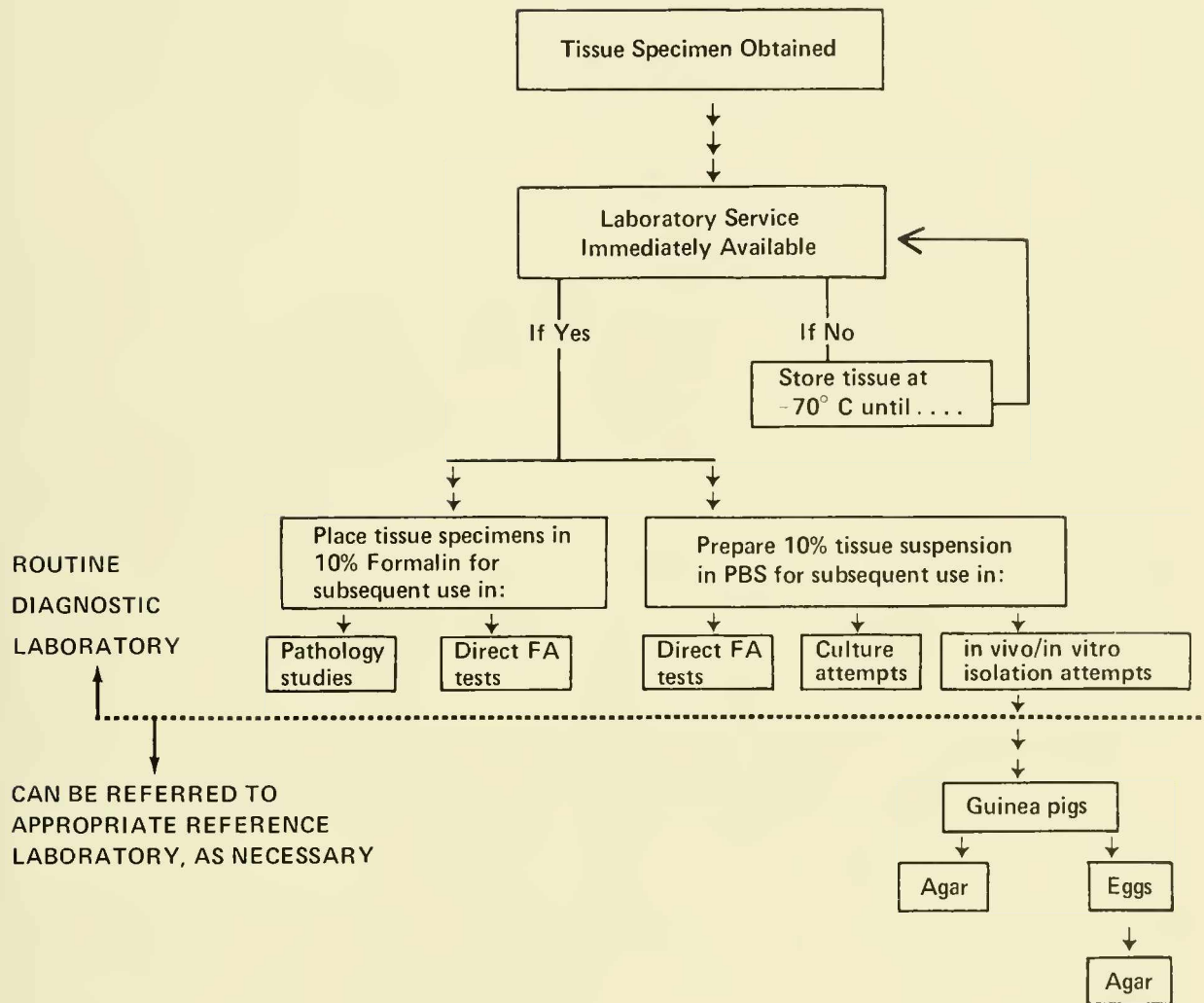


Figure 1. Treatment of Tissue Specimens for Isolation of the Legionnaires' Disease Bacterium



Suspend the minced tissue in enough phosphate buffered saline (PBS), pH 7.2, to form a 10% suspension. Inoculate aliquots of the suspension onto bacteriologic media which have been successfully used in growing the LDB and onto other bacteriologic media such as blood agar or Trypticase soy (TS) agar to serve as controls. Place other aliquots of the suspension on glass slides, air dry them, fix them in acetone or 10% neutral Formalin, and evaluate them with the direct FA test. Dispense 1-ml aliquots of the rest of the suspension into labelled 13- X 100-mm screw-capped test tubes; flash-freeze the samples in a dry ice-alcohol bath, and store them at  $-70^{\circ}\text{C}$  as reference materials.

## B. Interpreting FA Results and Direct Culture Attempts

The four possible combinations of results can be described as follows:

1. Both the FA results and attempts to culture the organism *in vitro* are negative. Such results indicate that the LDB is not present in the specimen, and attempts to isolate other pathogens should be considered.
2. Both the direct FA results and the *in vitro* isolation attempts are positive. Such results strongly indicate that the LDB is present in the specimen and that it may have been isolated. However, confirmational testing must be done to verify that the organism in question is the LDB; i.e., the bacterium isolated on agar should have cultural and staining characteristics and direct FA results comparable to those of prototype strains of the LDB.
3. Direct FA test results for tissue suspensions are negative, but cultural results are positive on media known to support the growth of the LDB. This combination of results must be interpreted cautiously. In some cases the LDB might be isolated directly on agar when it cannot be demonstrated with direct FA tests of the tissues. The resolution of such a situation led to the discovery of the second serogroup of LDB (8, 10). However, a more likely explanation for this conflicting pattern of direct FA and culture results is that a bacterium other than the LDB has been isolated. In any case, the reason for the discrepant results must be determined and the data reconciled by appropriate staining, FA, and cultural techniques before isolates are reported to be LDB.
4. Direct FA test results are positive for the LDB, but the organism is not isolated in direct cultures. If this combination of results occurs, attempts should be made to isolate the organism with guinea pigs and embryonated eggs as previously discussed and as described below.

## C. Inoculating Guinea Pigs

1. Prebleed each guinea pig and store the serum for future reference. Thaw an aliquot of the tissue suspension which was stored at  $-70^{\circ}\text{C}$ , and dilute it to approximately 5 ml in PBS. Inoculate (25-gauge needle) each of four adult male guinea pigs (approximately 600 g) intraperitoneally with a 1-ml aliquot of the tissue suspension.
2. Observe the guinea pigs daily for signs of illness. Symptoms sometimes include ruffled fur, watery eyes, and prostration. Determine their rectal temperatures at a specified time each day for 10 days. Temperatures  $\geq 39.5^{\circ}\text{C}$  are considered to be frank fevers.
3. Sacrifice febrile guinea pigs on the second day they have frank fevers by exposing them to excessive levels of  $\text{CO}_2$  vapor. Aseptically remove a portion of each guinea pig's spleen with sterile forceps and scissors. (Use one set of forceps and scissors to open the animal and a second set to remove the spleen.) Grind a piece of the spleen with alundum and dilute in PBS to form a 10% suspension. Dispense aliquots of the guinea pig spleen into labelled test

tubes and store as described above. Save one aliquot to be inoculated into embryonated eggs.\* Any remaining guinea pigs should be allowed to convalesce and then should be bled by cardiac puncture approximately 4 weeks after they were inoculated with the tissue suspension in order to obtain sera for serologic tests.

#### D. Inoculating Embryonated Eggs

1. Select twelve, 6- to 7-day-old embryonated hens' eggs from antibiotic-free flocks. Candle the eggs just before inoculating them to confirm that they are healthy. Label the eggs from 1 through 12 with a soft-lead pencil.
2. Working in a biological safety cabinet, dilute a 1-ml aliquot of the 10% guinea pig spleen suspension with 9 ml of PBS to form a 1% suspension. Draw up 6 ml of the 1% suspension into a 6-ml syringe fitted with a 20-gauge, 1½" needle. Cover the needle with the shield.
3. Disinfect the top of each egg with 70% ethanol (or tincture of Merthiolate). Allow the disinfectant to air dry. Disinfect an egg punch by dipping it in 70% alcohol and passing it through a flame. Use it to puncture the top of each egg. Inoculate each egg with 0.5 ml of the 1% guinea pig spleen suspension. (Be sure to insert the needle completely into the egg.) Seal the eggs with Duco Cement or its equivalent, and then incubate them at 35°-37° C in a humid incubator for 10 days.
4. Candle the eggs daily and discard all that die within the first 3 days after inoculation. Harvest eggs that die between the 4th and 10th days postinoculation on the day they die to obtain infected yolk sacs.

#### E. Harvesting Infected Yolk Sacs

1. Disinfect the top of an egg with 70% ethanol.
2. Disinfect a pair of blunt forceps by dipping them in 70% ethanol and passing them through a flame. Crack the shell of the egg with one prong of the forceps, and remove the uppermost quarter of the shell with the forceps.
3. Disinfect a second pair of blunt forceps as above. Use them to separate the yolk sac membrane from the other membranes; remove the yolk sac membrane, and place it in a sterile petri dish. Aseptically tear off a small piece of the membrane and drop it onto a 2" x 2" sterile gauze pad. Place the remainder of the yolk sac into a sterile 13- X 100-mm screw-capped test tube labelled with the appropriate egg number and date.
4. Blot the excess yolk from the small piece of yolk sac membrane on the gauze pad with another pad. Hold the tissue with sterile forceps, and use it to make smears on glass slides. Label each slide with the appropriate egg number and date. (Be sure that a particular egg, the smears of its yolk sac tissue, and the test tube used to store that yolk sac are assigned the same numbers.)
5. Wipe the excess tissue from the forceps with a clean 2" x 2" gauze pad, disinfect the forceps as above, and continue to harvest any remaining infected yolk sacs as instructed.
6. Flash-freeze the yolk sacs in a dry ice-alcohol bath, and store at -70° C.

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\*If embryonated eggs of the proper age are not available at the time the guinea pigs are sacrificed, guinea pig spleen suspensions can be quick-frozen and stored at -70° C until such eggs are available.



**F. Staining Smears**

1. Allow the yolk sac smears to air dry. Heat fix.
2. Stain the smears by the Giménez method as follows:
  - a. Filter working carbol fuchsin onto slide (through Whatman No. 2 filter paper) and let stand 1 to 2 min.
  - b. Wash slide thoroughly with tap water over sink.
  - c. Cover smear with malachite green for 6 to 9 sec.
  - d. Wash slide thoroughly with tap water.
  - e. Cover smear a second time with malachite green for 6 to 9 sec.
  - f. Wash thoroughly with tap water.
  - g. Blot slide dry and examine stained yolk sac smears under an oil immersion objective with light microscopy.
3. In properly prepared slides, the LDB stains red, and the yolk sac tissue appears as blue-green background cells.

**G. Confirming that Isolates are Legionnaires' Disease Bacterium**

1. After examining the Gimenez-stained yolk sac smears, select infected yolk sacs for further study that contain 50-100 organisms/field (1000X).
2. Thaw the test tube containing the selected yolk sac by placing it under running tap water.
3. Remove the yolk sac from the tube, aseptically grind it with alundum, and mix it with sterile PBS to form a 5% suspension.
4. Inoculate aliquots of the suspension onto TS agar, blood agar, F-G agar, and CYE agar (see Feeley et al., this manual). Incubate F-G agar cultures at 35°-37° C under 2.5% CO<sub>2</sub>. Incubate the other types of cultures at 35°-37° C (not in a candle jar). Incubate all cultures for 10 days.
5. Test an aliquot of the yolk sac suspension by direct FA to determine whether the isolate is an LDB. Draw the rest of the yolk sac suspension up into a Pasteur pipette, dispense it into sterile, 1-dram vials, and store them at -70° C as reference material.
6. The LDB should grow on F-G or CYE agar, but not on the other media listed above. Growth observed on the other media represents isolates other than LDB. In order to give a definitive report of the presence of the LDB, the isolate's growth and morphological properties must be confirmed as characteristic for the LDB, and its serologic reactivity in direct FA tests with standard conjugates to the LDB must be appropriate.
7. Bleed all surviving guinea pigs by cardiac puncture (with a 12-ml syringe and an 18-gauge 1½" needle) approximately 1 month postinoculation, and process the blood to obtain sera. Test these sera, and the sera obtained prior to inoculation, for reactivity with standard LDB antigens in indirect FA tests. Even if the LDB has not been successfully isolated with the procedures already described, the seroconversion of guinea pigs to a standard LDB antigen indicates that the LDB was present in the original patient sample that was submitted for analysis.



## ADDENDUM

## REAGENTS FOR GIMENEZ STAIN

## 1. Carbol basic fuchsin stock solution:

- a. 10% basic fuchsin in 95% ethanol \_\_\_\_\_ 100 ml  
 b. 4% aqueous phenol \_\_\_\_\_ 250 ml  
     (10 ml phenol in 250 ml distilled H<sub>2</sub>O)  
 c. distilled H<sub>2</sub>O \_\_\_\_\_ 650 ml  
 Mix well; incubate at 37° C for 48 h before use.

## 2. Stock buffers:

- a. 0.2 M NaH<sub>2</sub>PO<sub>4</sub> \_\_\_\_\_ 2.84 gm in 100 ml distilled water  
 b. 0.2 M Na<sub>2</sub>HPO<sub>4</sub> \_\_\_\_\_ 2.76 gm in 100 ml distilled water

## 3. Buffer solution:

- a. 0.2 M NaH<sub>2</sub>PO<sub>4</sub> \_\_\_\_\_ 3.5 ml  
 b. 0.2 M Na<sub>2</sub>HPO<sub>4</sub> \_\_\_\_\_ 15.5 ml  
 c. distilled water \_\_\_\_\_ 19.0 ml

## 4. Carbol basic fuchsin working solution:

- a. Carbol basic fuchsin stock \_\_\_\_\_ 4 ml  
 b. 0.1 M sodium phosphate buffer solution, pH 7.45 \_\_\_\_\_ 10 ml  
 c. Filter immediately and again before each use. Remains suitable for use for about 48 h.

## 5. Malachite green oxalite:

0.8% solution in distilled water

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# Primary Isolation Media and Methods

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## Primary Isolation Media and Methods

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Rickettsial techniques were used by McDade et al. in the initial isolation of the Legionnaires' disease bacterium (LDB) (4). In attempts to grow the LDB on artificial media, Dr. Robert Weaver inoculated portions of suspensions of LDB-infected embryonated hens' egg yolk sacs obtained from Drs. McDade and Shepard onto 17 different bacteriological agars. Of the media tested, the only one that supported growth of the LDB was Mueller-Hinton agar, supplemented with 1% hemoglobin and 1% IsoVitaleX (MH-1H) (1).

After studying MH-1H agar, we developed two solid media that better support the growth of the LDB from tissue. We also developed a diphasic medium for culturing blood. All these media—Feeley-Gorman (F-G) agar (1), charcoal yeast extract (CYE) agar (2), and CYE diphasic blood culture medium (3)—contain chemicals necessary for growing the LDB. Soluble ferric pyrophosphate or ferric nitrate replaces hemoglobin, and L-cysteine HCl replaces IsoVitaleX in these new media.

The CYE agar is the most sensitive artificial medium developed to date for isolating the LDB, but it does not offer the differential clues for LDB colony identification that the F-G agar allows. Consequently, we recommend that both CYE and F-G agars be prepared as described below and used in combination. Although we no longer use MH-1H agar for isolating the LDB, we include the instructions for preparing it as a reference. For blood cultures, we recommend a CYE diphasic medium.

### Preparation of Media

#### I. MH-1H Agar

Component A	Mueller-Hinton agar. . . . .	38 gm
	Distilled water. . . . .	490 ml
Component B	Hemoglobin powder . . . . .	10 gm
	Distilled water. . . . .	490 ml
Component C	IsoVitaleX (#11876). . . . .	20 ml

Prepare components A and B separately, and autoclave at 121°C for 15 min; cool to 50°C, combine A and B, and hold at 50°C in a water bath. Prepare Component C by adding 10 ml of sterile distilled water to each of two vials containing lyophilized IsoVitaleX. Add the contents of both vials to the A-B mixture. Before pouring 20-ml quantities of medium into each of a series of plastic dishes (15- X 100-mm), adjust the pH so that the final pH of the cooled medium will be 6.9. Allow agar plates to cool to room temperature. Measure the final pH of a plate of cool agar (procedure in the Quality Control section of this chapter). **The final pH must be between 6.85 and 6.95 in order to consistently support growth of the LDB.**

**II. F-G Agar**

Casein (acid hydrolysis) .....	17.5	gm
Beef extractives.....	3.0	gm
Starch .....	1.5	gm
Agar .....	17.0	gm
L-cysteine HCl · H <sub>2</sub> O.....	0.4	gm
Ferric pyrophosphate, soluble*.....	0.25	gm
Distilled water.....	1.0	liter

\* Available on request from Dr. Morris Suggs, Director, Biological Products Division, Center for Disease Control, Atlanta, Georgia 30333.

Baltimore Biological Laboratories (BBL) Mueller-Hinton (M-H) agar, lot #105621, contains all the ingredients of F-G agar except for L-cysteine HCl and ferric pyrophosphate, soluble, and provides a source of casein, beef extractives, starch, and agar. (NOTE: Other lots of M-H agar may vary too widely in formulation and stability to serve as the base for satisfactory F-G agar.)

Add the casein (acid hydrolysate), beef extractives, starch, and agar (or their equivalent—38 gm of M-H agar) to 980 ml of distilled water, autoclave at 121°C for 15 min, cool to 50°C in a water bath, and hold until L-cysteine HCl and soluble ferric pyrophosphate are added.

Prepare separate fresh solutions of L-cysteine HCl (0.4 gm in 10 ml distilled water) and soluble ferric pyrophosphate (0.25 gm in 10 ml distilled water). **Note the precaution for ferric pyrophosphate stated below.** Filter sterilize each solution separately. Add the L-cysteine HCl to the agar mixture first, and then add the ferric pyrophosphate.

Before pouring the warm medium into petri plates, adjust its pH so that the final pH of the cooled medium will be 6.9. Pour 20 ml into each of a series of plastic petri dishes (15- X 100-mm). Allow agar plates to cool to room temperature. Measure the pH of a plate of cool agar (procedure in the Quality Control section of this chapter). **The final pH must be 6.85 to 6.95 to consistently support growth of the LDB.**

**Precaution:** Soluble ferric pyrophosphate must be kept dry and stored in the dark; it is no longer usable if its color changes from green to yellow or brown. Prepare fresh solution of the compound each time it is needed for media. Do not heat over 60°C to dissolve. The mixture can be readily dissolved by placing in a 50°C water bath.

**III. CYE Agar**

Yeast extract (DIFCO) .....	10.0	gm
Activated charcoal (Norit SG)*.....	2.0	gm
L-cysteine HCl · H <sub>2</sub> O .....	0.4	gm
Ferric pyrophosphate, soluble.....	0.25	gm
Agar (DIFCO).....	17.0	gm
Distilled water.....	1.0	liter

Add all other ingredients of CYE agar except L-cysteine HCl and soluble ferric pyrophosphate to 980 ml of distilled water; dissolve by boiling; autoclave at 121°C for 15 min, and cool to 50°C in a water bath.

Prepare separate fresh solutions of L-cysteine HCl (0.40 gm in 10 ml distilled water) and soluble ferric pyrophosphate (0.25 gm in 10 ml distilled water). Filter sterilize each solution separately. Add the L-cysteine HCl to the basal medium first, and then add the ferric pyrophosphate. **Note the above precaution for ferric pyrophosphate.**

\* Available through Sigma Chemical Co., St. Louis, MO., Catalogue # C5510. Activated charcoal, washed with phosphoric and sulfuric acids.



Adjust the complete medium to exactly pH 6.9 at 50°C by adding 4.0 to 4.5 ml of 1.0 N KOH. The pH of this medium is critical.

Pour 20-ml quantities of medium into sterile petri dishes (15- X 100-mm). Swirl the medium between pouring plates to keep charcoal particles suspended.

#### IV. CYE Diphasic Blood Culture Medium

##### Agar Phase:

Activated charcoal (Norit SG).....	2.0 gm
Agar (DIFCO).....	17.0 gm
*Distilled water.....	500.0 ml

##### Broth Phase:

Yeast extract (DIFCO) .....	20.0 gm
L-cysteine HCl · H <sub>2</sub> O .....	0.40 gm
Fe(NO <sub>3</sub> ) <sub>3</sub> · 9H <sub>2</sub> O.....	0.10 gm
Distilled water.....	500.0 ml

Prepare agar first. Combine ingredients, boil, and dispense as 20-ml aliquots into each of a series of 125-ml Wheaton serum bottles. Stopper each bottle loosely with both a rubber stopper and a metal cap. Autoclave the bottles at 121°C for 20 min. cool to 50°C, remix the warm charcoal and agar thoroughly, and place the bottles at an angle so that an agar slant with a vertical height of 6.0 cm is formed. This procedure should leave a portion of the agar protruding above the 25 ml of liquid—broth plus specimen—that are added later.

Prepare the broth by first autoclaving the yeast extract and water at 121°C for 15 min. Allow to cool, and then add fresh sterile solutions of L-cysteine HCl and ferric nitrate in that order. Adjust the pH to 6.9 by adding 6.0 ml of 1 N KOH. Dispense the broth in 20-ml aliquots into the Wheaton bottles of charcoal agar slants. Seal the bottles by crimping the metal caps over the rubber stoppers. Check for sterility by preincubation at 35°C for 2 days.

#### Quality Control

Each batch of media must be tested for pH and ability to support the growth of the LDB. Ideally, all media should be tested with a standardized tissue inoculum. However, since this material is not generally available, stock culture inoculum is recommended with the understanding that it is a minimal quality control test.

1. The pH of the media must be  $6.9 \pm 0.05$ . Measure with a pH meter. To check solid media, use a surface electrode (if available), or emulsify the agar from one plate in distilled water, and measure the pH of the emulsion. **The pH is critical.** If given lots of ingredients do not conform to the acceptable pH range, adjust the pH of the complete liquid medium by adding 1 N HCl or 1 N KOH.
2. Check for support of growth in the following manner:
  - a. Prepare a standardized inoculum by emulsifying some actively growing LDB in sterile distilled water to a turbidity of a McFarland #4 standard.
  - b. Seed the media with 0.05 ml of the standard inoculum. Streak the plates for isolated colonies, and incubate at 35°C aerobically (except for F-G agar, which is incubated in air plus 2.5% CO<sub>2</sub>).
  - c. Examine the media daily. On agar plates, growth should be present in the heavily inoculated area after 2 days. Isolated colonies should be macroscopically visible in 4 to 5



days. Hold blood bottles for 2 wk, and tilt once each day so that the agar slant is inoculated with any growth in the broth. Look for turbidity of the broth and growth on the slant protruding from the broth.

3. Store media in the dark, in sealed plastic containers, in a refrigerator. (Media have been used successfully for growing the LDB after being stored for as long as 2 mon under these conditions.)

### Maintenance of Stock Cultures

Stock cultures of the LDB have remained viable for several months in our laboratory when stored as follows:

1. Prepare CYE agar and pour (5 ml/tube) into 16- X 125-mm screw-capped tubes. Allow to cool in a slanted position.
2. Inoculate the surface of the CYE agar slants with strains of the LDB.
3. Incubate the slants at 35°C for 2 days.
4. Remove, tighten the screw caps, and store in the dark at room temperature.

### Clinical Specimens

Clinical specimens can be either liquid or solid. The initial preparation, media inoculation, and subculturing of colonies thought to be LDB must all be done in a biological safety cabinet by personnel protected with masks and gloves.

#### I. Initial Processing and Media Inoculation

A. Liquid specimens (pleural fluid, transtracheal aspirate, sputum, blood, etc.) require no special preparation before being inoculated directly on media. Inoculate each container of F-G and CYE agar in two spots. Leave one spot undisturbed, and streak the other with a bacteriological loop for isolated colonies. Inoculate blood specimens directly into bottles of CYE diphasic medium. Use a sterile needle and syringe to put 5 ml of fresh blood into each bottle; mix well.

B. Solid specimens (lung, spleen, liver) should be processed (sliced or ground) as follows:

1. To prepare sliced specimens, cut the tissue with a sterile scalpel, and slide the freshly cut surface of the tissue over the surfaces of media to be inoculated. This procedure must be followed for tissues obtained from patients who received extensive antibiotic therapy.
2. Prepare 10% suspensions of ground tissue by emulsifying approximately 1.0 gm of tissue in 9.0 ml of sterile phosphate buffered saline (PBS), pH 7.2. Use a sterile mortar and pestle with sterile 60-mesh alundum as an abrasive. Mix well, and dispense into several sterile screw-capped test tubes. Use one aliquot for steps 3 and 4. Quick freeze the remainder for future use.
3. Inoculate CYE and F-G agars with one aliquot of the unfrozen suspension as described for liquid specimens.
4. Also, dilute the above 10% tissue suspension 1:50. Inoculate CYE and F-G agars with 0.1 ml of this 0.2% suspension. Spread the inoculum over the entire surface of each medium with a sterile glass spreading rod.

C. Incubate all inoculated CYE media aerobically in a humidified atmosphere at 35°C. Incubate the inoculated F-G agar at 35°C in air plus 2.5% CO<sub>2</sub>.

## II. Examination of Cultures

Examine all cultures macroscopically and microscopically each day.

### A. MH-1H Agar:

Examine plates for macroscopic growth. Colonies which are visible before 3 days of incubation probably are not LDB, because colonies of LDB do not usually appear on this medium before 5 days of incubation. Examine colonies suspected of being LDB with a dissecting microscope. They should have a cut-glass appearance, and there should be a distinctive brownish darkening of the surrounding agar.

### B. F-G Agar:

Examine this medium macroscopically with a desk lamp, allowing the light to pass through the agar. In areas of heavy inoculum, confluent growth will be visible in 2 to 3 days, and there will be a distinctive brownish darkening of the colonies and the surrounding agar. In lightly inoculated areas, colonies of LDB should appear in 4 to 5 days as white pin-point dots. These colonies grow larger after longer incubation. Examine heavy growth in the dark with a long-wave (366 nm) ultraviolet light: a butter-yellow color should be detectable in the 4- to 5-day growth. Examine suspected LDB colonies microscopically by placing plates on the stage of a dissecting microscope and illuminating from one side with a light source held at an angle slightly  $>10^\circ$  to the horizontal. LDB colonies will have a cut-glass texture.

### C. CYE Agar:

Examine CYE plates as described for F-G plates. LDB colonies will appear in 3 to 5 days. Young colonies have a slight cut-glass texture which is rapidly lost after additional incubation. The browning of the agar is not visible on this black medium.

### D. CYE Diphasic Blood Culture Medium:

Examine bottles daily for growth on the portion of agar slant extending above the broth. Reinoculate this part of the slant daily by tilting the bottle so that broth flows over it. Growth should be observed in about 6 to 8 days. Subculture colonies to CYE agar. Keep bottles for 14 days before discarding as negative.

## III. Presumptive Identification of LDB Colonies

Subculture colonies with a characteristic cut-glass appearance to both LDB-growth-supporting media (F-G and CYE agar) and non-LDB growth-supporting medium (a blood agar plate that does not contain L-cysteine). Incubate media at  $35^\circ\text{C}$  in air plus 2.5%  $\text{CO}_2$  for 5 days. Examine plates daily for growth. Test all cultures that grow on F-G and/or CYE agar but not on blood agar. *Cultures which grow on plain blood agar are not the LDB.* A culture that has typical morphological and biochemical characteristics, a compatible cellular fatty acid profile, and a high DNA-relatedness value to LDB strain Philadelphia 1 is the LDB.

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**Figure 1:** Growth of the Legionnaires' disease bacterium (LDB). Suspensions of guinea pig spleen tissue infected with LDB strain Philadelphia 1 were inoculated onto CYE, F-G, and MH-1H agars. MH-1H and F-G agars received a 1/5,000 dilution; CYE agars received a 1/500,000 dilution. Plates were incubated at  $35^\circ\text{C}$  in air + 2.5%  $\text{CO}_2$ . Pictures show growth at 4, 6 and 10 days on CYE (black plates), F-G (clear plates), and MH-1H (red plates) media.

**Figure 2:** Heavy growth of the LDB on F-G agar showing brown pigment production.

**Figure 3:** The LDB on F-G agar showing cut-glass appearance of colonies resulting from oblique lighting.

**Figure 4:** CYE diphasic blood culture medium; Wheaton bottle with a charcoal agar slant and broth inoculated with blood.





Figure 1.



Figure 2.



Figure 3.



Figure 4.



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# Method for Isolating Legionnaires' Disease Bacterium from Soil and Water Samples

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# Method for Isolating Legionnaires' Disease Bacterium from Soil and Water Samples

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## INTRODUCTION

Thirteen documented outbreaks of Legionnaires' disease (LD) have occurred in the United States since 1965 (2). Epidemiologic investigations in several outbreaks indicated that the etiologic agent was transmitted in air. Among the environmental sources implicated were water from air-conditioning cooling towers or evaporative condensers (3) and soils disrupted by construction work and presumably transported as dust by air currents (1,6). In order to document environmental sources of the Legionnaires' disease bacterium (LDB) microbiologically, we modified the primary isolation system that utilizes guinea pigs and embryonated eggs (described by McDade in this manual). We used these procedures to isolate the LDB from 38 of 176 environmental samples of water and soil collected during 11 epidemic investigations in 10 states (5, unpublished data). All samples were subjected to two types of analyses: (a) primary evaluation using the direct fluorescent antibody (FA) test and culture media and (b) guinea pig inoculation and evaluation.

## COLLECTION AND PRIMARY ANALYSIS OF SAMPLES

Water or soil and other solid samples should be collected aseptically in chemically-inert sterile containers and refrigerated until analyzed. Personnel analyzing these samples should wear proper safety attire and manipulate the materials only in a biological safety cabinet.

Primary analysis involves the direct FA test and primary culture on Feeley-Gorman (F-G) and charcoal yeast extract (CYE) agars. The media are described by Feeley et al. in this manual.

**Direct FA.** Screen samples with direct FA to determine which contain bacteria which stain with the LDB conjugate(s) and are thus most likely to yield isolates of the LDB when cultured. Prepare smears of water samples and aqueous suspensions of soil and other solid material by completely filling the circles on a microscope slide with the sample. Let the slides air dry, fix with gentle heat and then with 10% neutral Formalin. The procedure is described fully by Cherry and McKinney in this manual.

Four serogroups of the LDB have been defined to date (4). Although ideally all specimens should be screened for the presence of all known serogroups, appropriate FA conjugates must be available in order to implement such a policy.

**Primary Culture of Water Samples.** Prepare a water sample for direct culture on media by making serial 10-fold dilutions to  $10^{-4}$  in sterile distilled water. Inoculate portions (0.1 ml) of the undiluted sample and of each dilution to individual F-G and CYE agar plates, in triplicate, and spread with a smooth glass rod. Incubate the plates at 35°C in air plus 2.5% CO<sub>2</sub>. Examine plates daily for growth. After 2 to 3 days of incubation, calculate colony forming unit (CFU) value for



growth on each medium, and record as follows:

sparse growth . . . . .	$<10^4$ CFU/ml
moderate growth. . . . .	$10^4$ to $10^6$ CFU/ml
heavy growth. . . . .	$>10^6$ CFU/ml

Use the calculated microbial density or CFU for each water sample as a guide to determine amount of sample to inject into guinea pigs in the procedure described in a later section of this chapter.

Examine plates at 3 days and daily thereafter for the development of additional colonies resembling the LDB. Colonies of the LDB are usually visible within 4-7 days; however, incubate plates with no growth or well-dispersed colonies of normal flora for 14 days before discarding as negative. Confirm isolates suspected of being LDB with tests described elsewhere in this manual.

**Primary Culture of Soil Samples.** Culture soil samples on F-G and CYE agar media. To prepare a soil sample for culture, first add 10 gm of soil to 100 ml of sterile distilled water containing 0.5% Tween 60. Then shake the suspension vigorously for 30 min on a mechanical shaker. Let the heavy particles settle for 5-10 min, and then plate the aqueous layer as described for liquid samples using serial 10-fold dilutions through  $10^{-4}$ . Examine all plates for LDB as described above for water samples.

## PREPARATION OF SAMPLE AND INOCULATION OF GUINEA PIGS

Although the guinea pig procedure is a laborious and tedious means of isolating the LDB, researchers have not yet found a satisfactory alternative for working with soil and water samples. Only once in our laboratories has the LDB been isolated directly from a water sample without inoculating guinea pigs. Results of direct FA tests (performed with polyvalent conjugates against all known serogroups) show which samples are most likely to contain the LDB when tested further. Environmental samples are frequently much more contaminated with other microflora than are clinical specimens. If a guinea pig is inoculated with a large number of microbial flora, the animal is likely to die of causes other than an LDB infection. Therefore, it is important to be aware of the normal flora concentration in the samples when choosing the inoculum for the guinea pigs. We currently do this by culture. Other potentially suitable methods for estimating microbial density of non-LDB which we have not yet evaluated are direct microscopic observation of Gram-stained slides and growth of non-LDB on total plate count media. Before injecting guinea pigs with samples, establish each animal's average baseline temperature from measurements taken on each of the 5 days before inoculation.

**Water Samples (See Figure 1).** If the CFU/ml described above from cultures of a water sample is less than  $10^4$ , concentrate the sample. Centrifuge 100 ml at high speed (ca. 2900 X g) for 30 min; discard the supernatant. Resuspend the sediment in 10 ml of sterile water. Mix well, and inoculate two guinea pigs intraperitoneally (IP) with 3 ml each of the sample. When the CFU/ml for a sample is moderate ( $10^4$  to  $10^6$ ), inject 3 ml of the untreated sample into each of two guinea pigs. If the CFU/ml for a sample is high ( $>10^6$ ), dilute it to  $10^6$  CFU/ml and inject 3 ml of the diluted sample.

**Soil Samples (See Figure 2).** Suspensions of soil samples to be used as inoculum are prepared as follows: Add 10 gm of soil sample to 100 ml of sterile distilled water containing 0.5% Tween 60. Place the mixture on a shaker at moderate speed for 30 min. Allow 5-10 min for the heaviest particles to settle, decant and save the top portion, and discard the heavy sediment. Centrifuge the top portion (approximately 90 ml) of the suspension at 400 X g for 5 min. Carefully remove the supernatant and discard the sediment. Centrifuge the supernatant at 2900 X g for 30 min. Discard this final supernatant, and resuspend the sediment in 10 ml of sterile distilled water.

Determine the total CFU/ml in the final suspension, adjust to  $10^6$  CFU/ml, and inject 3 ml into each of two guinea pigs. Refrigerate the final suspension for 2 to 3 days until bacterial quantitation is completed before inoculating the guinea pigs.

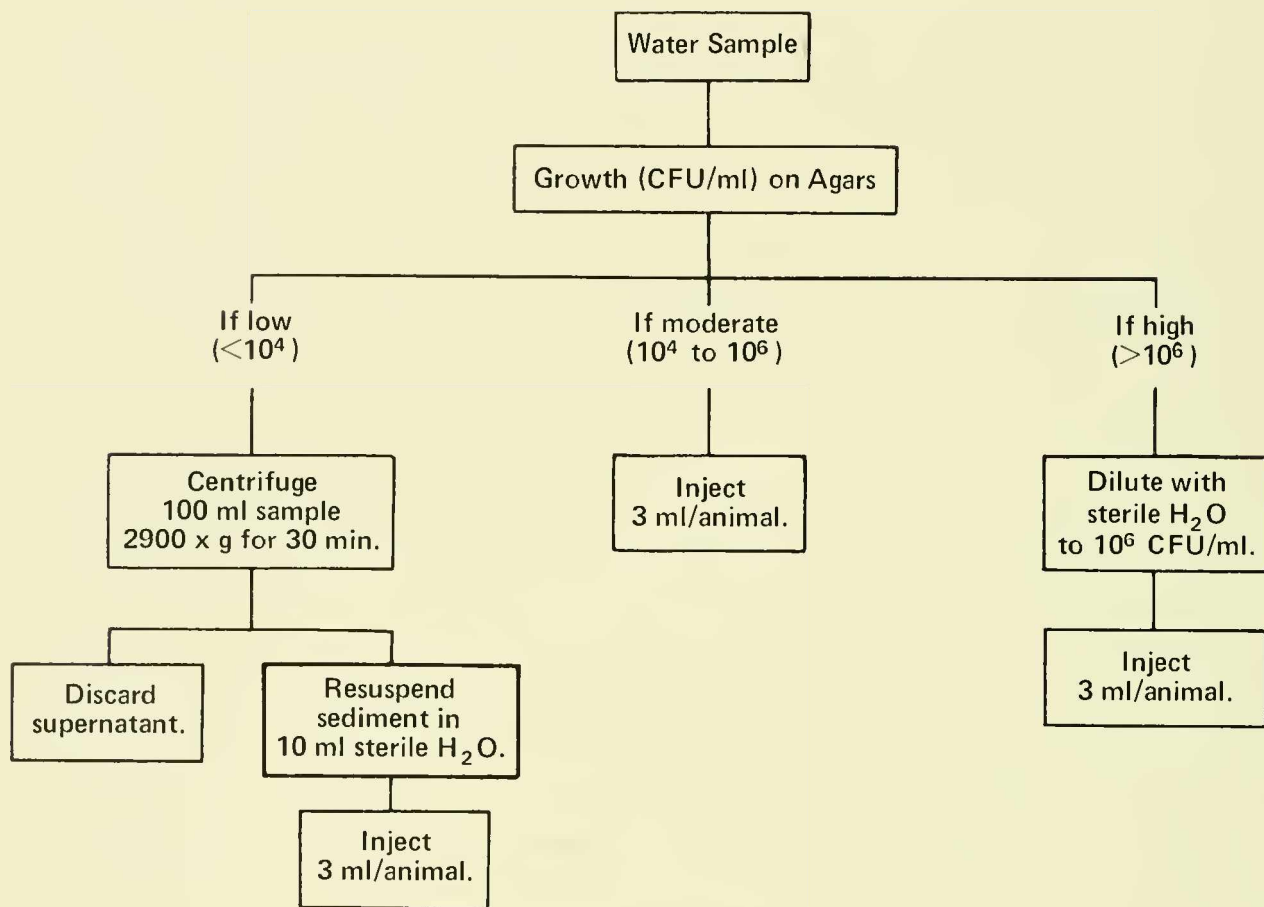


Figure 1. Preparation of Water Samples for Guinea Pig Inoculation

**Observing the Inoculated Guinea Pigs and Collecting Spleens.** Measure the temperature of the inoculated guinea pigs at a predetermined time each day for 7 days, and observe the animals for signs of illness, i.e., ruffled fur, water eyes, prostration, and hypothermia. If there is a rise in temperature of 0.6°C for 2 consecutive days or any rise in temperature with 1 of the above symptoms, sacrifice and examine the affected animal. Sacrifice and examine all other guinea pigs in the study at 7 days. Aseptically remove splenic tissue from the animals for culturing. Culture peritoneal exudates and material swabbed from peritoneal walls by direct plating on CYE and F-G agars.

## CULTURING GUINEA PIG SPLENIC TISSUE

The procedures for preparing tissue homogenates, inoculating embryonated eggs, and collecting yolk sacs are described by McDade in this manual. Splenic tissue and yolk sacs are inoculated to F-G and CYE agar according to methods described by Feeley et al. in this manual. Isolates suspected of being LDB must be confirmed as such with tests described elsewhere in this manual.

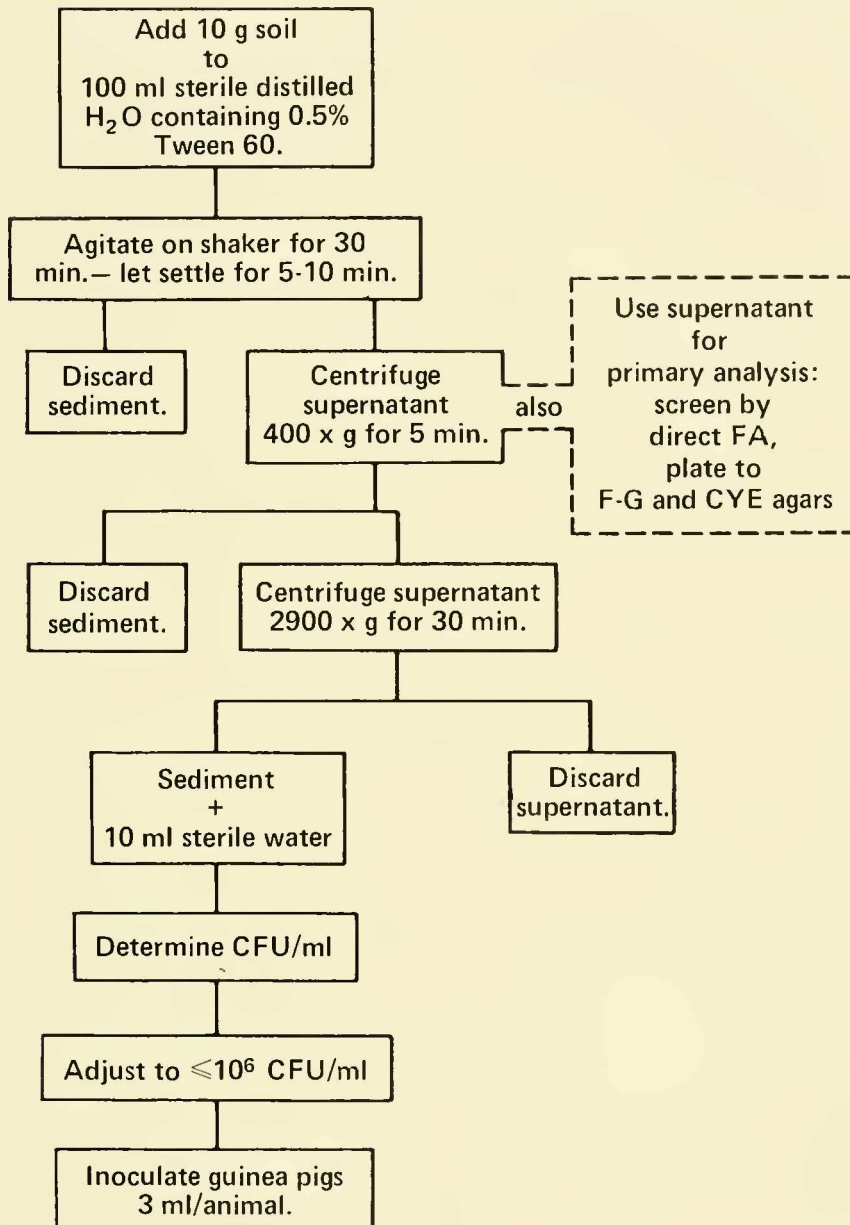


Figure 2. Preparation of Soil Samples for Guinea Pig Inoculation



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# Detection of Legionnaires' Disease Bacteria in Clinical Specimens by Direct Immunofluorescence

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# Detection of Legionnaires' Disease Bacteria in Clinical Specimens by Direct Immunofluorescence

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## INTRODUCTION

Direct fluorescent antibody (FA) staining is useful for detecting Legionnaires' disease bacteria (LDB) in clinical specimens and in environmental samples. In early studies of the LDB, a fluorescein isothiocyanate (FITC) conjugate of rabbit antibodies to the Knoxville 1 strain stained all of 13 available isolates (3, 4). LDB isolates have since been obtained that do not stain with the Knoxville 1 conjugate. Four serogroups of LDB have been recognized to date by direct FA staining. Knoxville 1, isolated by McDade from a postmortem lung specimen from a patient in Knoxville, Tennessee, is representative of serogroup 1; Togus 1, isolated by McDade from a postmortem lung specimen from a patient in Togus, Maine, is representative of serogroup 2 (9); Bloomington 2, an environmental isolate obtained from a creek in Bloomington, Indiana (2, 11), is representative of serogroup 3; Los Angeles 1, obtained from a human lung specimen (5) is representative of serogroup 4. Serogroup-specific FA conjugates for each of the four recognized serogroups (10), and a polyvalent conjugate containing all four group-specific conjugates have been prepared. The current practice in diagnostic direct FA staining at the Center for Disease Control (CDC) is to first test clinical specimens by staining with the polyvalent reagent. If the tissue is FA positive, the serogroup is determined by staining with each of the four group-specific conjugates. Although the great majority of FA positive clinical specimens tested at the CDC have reacted only to serogroup 1 conjugate, four clinical specimens have been found positive only with serogroup 2 conjugate, three only with serogroup 3 conjugate, and two only with serogroup 4 conjugate. It is likely that additional serogroups of LDB will be recognized in the future. Therefore, when isolates are obtained that have the growth characteristics and colonial appearance of LDB but do not stain with polyvalent conjugate, further definitive tests for LDB such as biochemical tests, gas-liquid chromatography of cellular fatty acids, and measurements of DNA relatedness must be performed.

Legionnaires' disease (LD) is naturally acquired by the respiratory route, and organs other than the lung rarely appear to be involved. Therefore, this chapter deals almost exclusively with detecting LDB in lung tissue or lung exudates. If all available clinical, epidemiological, microbiological, and serological data are considered in interpreting direct FA test results, they are extremely valuable for indicating the scope of an outbreak and for establishing retrospective and current diagnoses.

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## PREPARATION OF SPECIMEN MATERIAL

### Tissue scrapings of Formalin-fixed tissue (4)

1. Select one or more areas of the lung or other tissue for testing. With lung tissue, choose dense areas of gray or reddish consolidation.
2. Transfer each tissue block to a sterile petri dish.
3. With a sharp scalpel, cut through these areas to produce new tissue faces for scraping.
4. Grasp the tissue with forceps, and holding the scalpel at a right angle to the tissue face, scrape it to produce a fine purée of tissue particles. The lung tissue of victims of Legionnaires' disease is usually quite friable. If the tissue is rubbery or spongy, a positive test is unlikely.
5. Using the scalpel blade, smear the particles of tissue and tissue fluids onto two 1.5-cm circles on each of three microscope slides. Stain one smear on the first slide with preimmune or normal conjugate and the other with the polyvalent conjugate. Reserve the other four smears for staining with the four monovalent serogroup conjugates if the polyvalent conjugate gives positive results.
6. Let the smears air dry. Gently heat fix. It is not necessary to remove the Formalin before staining.

### Fresh or fresh-frozen tissue (process in a safety cabinet)

When fresh or fresh-frozen tissue is obtained through autopsy or biopsy, the first laboratory procedure should be to inoculate culture media for attempts to isolate the LDB. This will minimize contamination of the tissue. Then select tissue for other tests, including direct FA.

1. Using sterile instruments, cut a fresh face of tissue; with the forceps, press and squeeze the tissue against three clean microscope slides, making impression smears within the two 1.5-cm circles on each slide. If the tissue is so moist that smears may be too thick, blot on sterile gauze or culture media before imprints are made.

Alternatively, grind the tissue with sterile phosphate buffered saline (PBS) and alundum in a sterile mortar, or homogenize the tissue in a Ten Broeck or comparable tissue grinder. Use sufficient PBS to give an approximate 10% tissue homogenate. Prepare smears in the same manner as described for Formalin-fixed tissue. With larger pieces of tissue this method produces a more representative sample and reduces sampling error.

2. Air dry and heat fix.
3. Fix smears for 10 min by covering them with 10% neutral Formalin. Do not let the Formalin dry on the smears.
4. Drain off the Formalin and gently and briefly dip each slide into distilled water to remove the remainder of the Formalin. (If slide is not rinsed, Formalin will not interfere with staining.)
5. Air dry.

### Tissue sections

Legionnaires' disease bacteria maintain their serologic integrity through histopathological processing and can easily be demonstrated in tissue sections if they are present in reasonable numbers. However, they are not as easily demonstrated in such sections as they are in scrapings of Formalin-fixed lung or imprints of fresh lung tissue because many of the bacteria are intracellular, lie at many different levels in the section, and are shrunk by the histological processing. Counterstains, such as rhodamine-labeled rabbit serum or rhodamine-labeled bovine serum albumin, make the organisms much more visible in tissue sections. (See Addendum at the end of

this chapter for counterstain preparation.) Evans' blue or other counterstains will also probably be helpful, but their relative value has not been sufficiently documented.

1. Cut tissue sections as thin as possible (4  $\mu$ m or less) from paraffin blocks.
2. Affix the sections to slides by heating them for approximately 15 min at 58° to 60°C.
3. Remove the paraffin and hydrate the tissue sections by sequentially dipping the slides in two changes each of xylene, absolute ethanol, 95% ethanol, and distilled water.
4. Air dry.

#### **Lung exudates and pleural fluids (process in a safety cabinet) (1, 6, 8)**

Expectorated sputa, transtracheal aspirates (TTA), bronchial washings, pleural fluids, and other specimens from the lower respiratory tract (LRT) are all satisfactory materials for study. Patients with Legionnaires' disease (LD) frequently do not produce much sputum, and what they produce may not be purulent. The secretions are usually extremely viscid and tenacious. Smears usually contain tissue cells which will autofluoresce or stain nonspecifically, so counterstains are needed. Until selective media are developed, it is probably not worthwhile to culture expectorated sputum; however, clean specimens such as TTA, bronchial washings, and pleural fluids that are taken by invasive procedures should be cultured. Several isolates have been obtained in this way.

1. Select a viscous portion of the sputum, aspirate, washing, or other LRT specimen, and prepare two smears of moderate thickness on each of three microscope slides. Pleural fluids tend to form a fibrin clot on the slide, and unless processed carefully, the entire film can be dislodged.
2. Air dry and heat fix.
3. Fix smears in 10% neutral Formalin for 10 min. Either place each slide in a separate coplin jar or lay each one flat and flood it, but **do not let the Formalin dry on the slide**.
4. Drain off the Formalin.
5. Air dry.

#### **Blood cultures**

Inoculate 5 ml of patient's blood into a bottle of charcoal yeast extract (CYE) diphasic blood culture medium (see chapter by Feeley et al., this manual). Incubate at 35°C aerobically. Each day flood the agar slope of the diphasic medium with the broth portion. Growth may not be apparent for four or more days. When growth is apparent on the agar medium, subculture to slants of CYE agar and Feeley-Gorman (F-G) agar. Prepare smears for direct FA, Gram's and other stains by standard and previously described methods.

#### **Culture smears (process in a safety cabinet)**

1. Make suspensions of known or suspected LDB cultures in 1% Formalin in 0.85% NaCl solution to give a light turbidity (McFarland No. 2).
2. Prepare smears on double-ring slides.
3. Air dry and heat fix.

#### **FA STAINING PROCEDURE**

1. Methods for preparing the specific LDB conjugate and a counterstain reagent are detailed in the Addendum to this chapter.



2. Stain all preparations by covering the smear nearest the labeled end of the slide with 1-2 drops (0.05 ml) of the preimmune or other control conjugate.
3. Cover the second smear with 1-2 drops of the use dilution of the polyvalent LDB conjugate.
4. Place the slides in a covered chamber to prevent evaporation during staining.
5. Stain for 20 min.
6. Remove excess conjugate by holding the slide level, but perpendicular, and tapping it against a towel. Quickly and gently rinse smears with a stream of PBS from a wash bottle while keeping the slide roughly horizontal with the long edge tipped slightly downward. Prevent the specific conjugate from coming into contact, even momentarily, with the control smears.
7. Immerse slide in PBS for 5 min. Use a separate PBS container for each slide to prevent cross-contamination with organisms that may wash off of LDB positive slides.
8. Rinse each slide in distilled water from a wash bottle.
9. Air dry.
10. Add a small drop of buffered glycerol (pH 9.0) and a 1 or 1½ (Corning) cover slip to the smears.

## EXAMINATION OF STAINED SLIDES

Examine first under the 10X objective of the fluorescence microscope. In strongly positive preparations the bacteria may be visible as uniformly sized dots. Select areas of the smear where organisms may be present, and switch to a 40X or 63X oil immersion objective for screening. Use the 100X oil objective for close study and confirmation. The bacteria will be visible as single short rods or small intracellular or extracellular clumps of organisms showing strong peripheral staining with darker centers. No specimen should be reported as negative until after at least a 5-min search.

If organisms of appropriate morphology are stained by the polyvalent LDB conjugate and not by the control conjugate, stain the additional smears with the LDB serogroup-specific reagents.

## CONTROLS

The most important controls consist either of preimmune conjugate from the same animals which furnished the specific reagent or conjugates prepared from the sera of unimmunized animals of the same species as those immunized. These control reagents should have approximately the same protein content and F/P ratio as the specific conjugate.

As controls, the conjugates should be used at either the same dilution as that of the specific conjugate, or preferably, at twice that concentration, as a margin of safety.

Each specimen preparation showing specific staining must be tested with the control reagent to insure that the observed reaction is serologically specific. Other essential controls are culture suspensions, and positive and negative preparations of tissue scrapings, tissue imprints, sections, and smears of lower respiratory tract secretions, depending upon the types of diagnostic material being submitted for examination.

Positive and negative smears should be carried through the specimen staining procedure each time a group of specimens is processed. Negative smears may be made from suspensions of any heterologous bacteria which are not related serologically to the LDB.



## INTERPRETATION OF RESULTS AND TEST LIMITATIONS

In all clinical specimens, except for lung exudates (LE), the following criteria are used to evaluate the test results:

Result	Report
≥25 strongly fluorescing bacteria/smear*	FA +
<25 strongly fluorescing bacteria/smear	#'s only
0 strongly fluorescing bacteria/smear	FA -

\*In many FA-positive specimens, an average of 50 or more LDB per field (1250X) may be observed.

Positive FA staining should be reported as FA+ (group 1, 2, 3, or 4) depending on which diagnostic conjugate gave positive FA staining. In LE, organisms are seldom numerous. Thus, the observation of more than five brightly stained small rods morphologically typical of the LDB constitutes a positive result in this type of specimen.

Interpreting the results of stained tissue scrapings, sections of Formalin-fixed lung, fresh lung imprints, cultures, and pleural fluids is rather straightforward (3, 4). Organisms in culture are usually longer rods than those seen in tissues. In older cultures, long filaments, swollen rods, and other bizarre forms may be seen.

Interpreting the results of stained LE specimens such as expectorated sputa, transtracheal aspirates, and those obtained by bronchial lavage or by bronchoscopy is more difficult. Tissue cells and white blood cells may be highly autofluorescent. Bacteria such as staphylococci, diplococci, and streptococci may fluoresce because of natural antibodies which are in the serum of the immunized rabbit. Frequently, many of the LDB are disintegrating from the effects of cellular defense mechanisms. Ragged, swollen, atypical forms and fluorescent debris are commonly observed. A smear stained with the negative control conjugate must be studied as carefully as one which is presumed to be positive. Familiarity with the morphology and staining characteristics of the LDB is imperative if false-positive reports are to be avoided. For example, one of 23 strains of *Pseudomonas fluorescens* tested was brightly and specifically stained by the working dilution of the serogroup 1 LDB conjugate. The fluorescent *Pseudomonas* rods may, however, appear wider than the LDB rods. The use of counterstains for sputum and tissue examination has proved very helpful.

Because of the size of the particles of tissue obtained by scraping Formalin-fixed lung, many particles are lost from the slide during processing. The free bacteria and tissue cells will, however, remain on the slide to give a good test substrate.

Although isolation and characterization of the LDB is discussed elsewhere in this Manual, we emphasize the necessity of attempting to culture these organisms from lung tissue, pleural fluid and LE of patients suspected of having the disease. Culture should be attempted even though no LDB-like bacteria are seen by direct FA staining. The organisms may be present in numbers too small to be detected by FA staining or new serogroups of the LDB may be producing infection. All LDB isolates should be fully characterized culturally, serologically, and physiologically to determine if they fit the accepted criteria for classification as the LDB.

## DISCUSSION

Recently, Lattimer, Rhodes, and Cepil (8) commented as follows: "the degree of fluorescence in the I.F.A. and D.F.A. tests used for diagnosing Legionnaires' disease is dependent not only on the presence of the bacterium but also on the materials from which the organism is isolated (lung, egg yolk sac, or artificial media) and by the method used to prepare the antigen. Until a standard antigen with defined sensitivity and specificity is developed, serodiagnosis should

be considered as only suggestive for the diagnosis of Legionnaires' disease and definitive diagnosis should be established by culture." In respect to the direct fluorescent antibody (D.F.A.) test, this view places unjustified constraints on the application of the test and limits its proven value. Our experience with large numbers of human and animal diagnostic specimens over the past 2 years supports the reliability of the test if the methodology presented in this chapter is used. In the hands of experienced workers, the specificity of the direct test is high. Failure to isolate LDB from clinical materials that are positive by direct FA staining does not necessarily indicate cross-reactivity. The best available media are probably still suboptimal for growth and the LDB are quite susceptible to inhibition by contaminants. The organisms may be drug inhibited or they may have lost viability during storage or handling of the specimens. If non-LDB are present and responsible for positive staining, they must be more difficult to isolate than the LDB because we have not encountered them during the examination of thousands of colonies from human and guinea pig tissues and from sputum and environmental specimens. We isolated only one organism, a single strain of *Ps. fluorescens*, that is antigenically related to the serogroup 1 LDB. It was present as a contaminant in commercial Evans' blue counterstain. Twenty-two laboratory-maintained strains of *Ps. fluorescens* were nonreactive with the group specific and polyvalent LDB conjugates. Over 400 pure cultures representing 25 genera and 60 species and 54 unidentified cultures were negative. Lung tissues from a number of patients with pneumonia known to be caused by other bacteria or fungi also were negative when tested with LDB conjugates.

Undoubtedly the future will reveal some additional antigenic relationships between LDB and other organisms. Certainly, every effort should be made to isolate and identify organisms which are stained by the LDB conjugates. However, sufficient experience has been accumulated attesting to the specificity of the direct FA test for the LDB to place the burden of proof of nonspecificity on those who suggest otherwise.

The fact remains that direct FA staining of LDB is still the only rapid diagnostic test for these organisms and also the only specific laboratory test that can be used by clinicians for guidance in instituting therapy. Autopsy and biopsy tissue can be screened for LDB more quickly by the direct FA test than by pathological examination. In addition the direct FA test has a dimension of serological specificity not possessed by the Dieterle silver impregnation stain and other histological staining techniques used to demonstrate the organisms in tissue.

## PERFORMANCE CHARACTERISTICS

1. The diagnostic dilution of an LDB polyvalent or monovalent conjugate should stain, at least to a 3+ intensity, all known strains of LDB belonging to the serogroups (polyvalent) or to a serogroup (monovalent).
2. The corresponding control conjugate at its use dilution should not stain any of the stock strains to more than a 1+ intensity.

## SUMMARY

In this chapter we discuss the basic steps required for detection of the LDB by direct immunofluorescence. The question of controls and the interpretation of results and limitations of the direct FA tests are also discussed. Questions related to specificity and sensitivity are treated in some detail. An Addendum includes the preparation of vaccine for antiserum production, the preparation of reagents, and a listing of LDB isolates by serogroup.

Finally, the color plate (Figures 1-8) shows the appearance of the LDB in pure culture at different ages, in Formalin-fixed tissue sections, in scrapings of wet Formalin-fixed tissue, and in sputum from LD patients.



### Legends for Figures

**Figure 1.** Smear of 48-h culture of LDB stained with a 1:80 dilution of homologous conjugate. Culture was grown on modified Mueller-Hinton medium and suspended in phosphate buffered saline containing 1% Formalin. Note that these cells resemble those seen in tissue. Photographed with a 100X oil objective on 135-mm Ektachrome 200 film. Magnification X400 as photographed.

**Figure 2.** Smear of 5-day-old culture of LDB grown on a different modification of Mueller-Hinton medium. Stained and photographed as in Figure 1.

**Figure 3.** Deparaffinized section of LDB-infected human lung tissue showing positive FA staining of serogroup 2 LDB with serogroup 2 conjugate. The Atlanta 1 isolate (serogroup 2) was obtained from a fresh-frozen specimen of the same lung tissue. Photographed with 25X objective.

**Figure 4.** Deparaffinized section of lung tissue showing negative FA staining with serogroup 1 conjugate on tissue infected with serogroup 2 LDB. The lung tissue was from the same paraffin-embedded block as that used in Figure 3. Photographed with 40X objective.

**Figure 5.** Deparaffinized section of heavily infected human lung tissue showing positive FA staining of LDB with serogroup 3 conjugate. The tissue was FA negative when stained with conjugates for serogroups 1,2, and 4. Photographed with 40X objective.

**Figure 6.** LDB as they appear in a smear of scrapings of wet Formalin-fixed human lung tissue. Note large number of extracellular bacteria, many of which have been released from broken tissue cells. Note absence of chains or filamentous forms. Stained with a 1:80 dilution of homologous conjugate. Photographed as in Figure 1.

**Figure 7.** LDB in sputum from a patient with LD. Stained with serogroup 1 conjugate. Photographed with 100X oil objective on 135-mm Ektachrome 400 film. Magnification X400 as photographed.

**Figure 8.** LDB in sputum from a patient with LD. Stained and photographed as in Figure 7. Note filamentous forms.





Figure 1.



Figure 2.



Figure 3.



Figure 4.

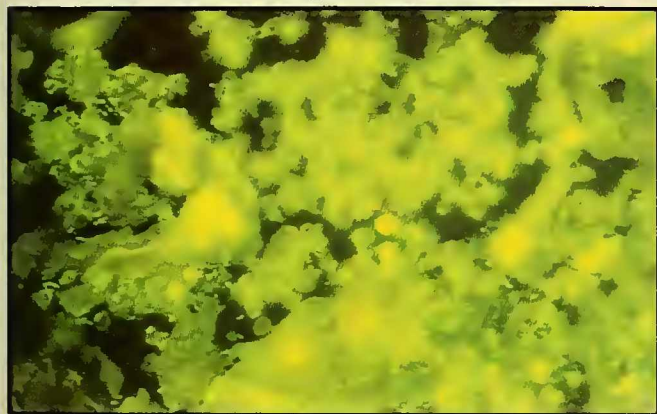


Figure 5.



Figure 6.

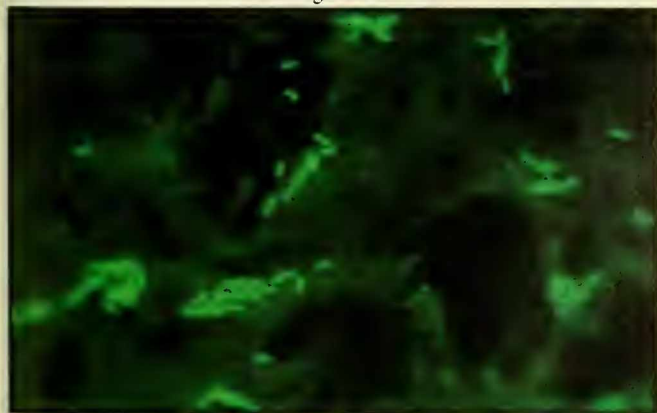


Figure 7.



Figure 8.

## ADDENDUM

### I. PREPARATION OF FLUORESCENT ANTIBODY REAGENTS

#### A. Production of Antisera

##### 1. Preparation of vaccine for antiserum production

The bacterial cell vaccine is prepared as a suspension of cells harvested from solid media, killed by suspending overnight in 1% Formalin-0.85% saline, centrifuged, and resuspended and adjusted with 0.5% Formalin-0.85% saline to give a turbidity reading of 40 International Units (Maaloe, O. 1955. The International Reference Preparation for Opacity – Notes and Description. Bull. World Health Organ. 12: 769-775). This is roughly comparable to  $4 \times 10^9$  bacterial cells/ml. For injection, a portion of the suspension is mixed with an equal volume of Freund's complete adjuvant.

##### 2. Immunization schedule

**Day 1** – Pre-bleed the rabbits for control sera. Distribute 2 ml of the cell suspension-Freund's adjuvant mixture by intracutaneous injection into 20-40 sites along the shaved back of each rabbit.

**Day 31** – Inject 1 ml of the cell suspension-Freund's adjuvant mixture deep into the thigh muscle of each hind leg of the rabbit (2 ml total).

**Day 38** – Take 50 or 60 ml of blood from the ear for antiserum. Inject 2 ml of the Formalinized cell suspension intravenously into each rabbit (no adjuvant).

**Day 45** – Take 50 or 60 ml of blood from the ear. Inject 2 ml of Formalinized cell suspension intravenously into each rabbit.

**Day 52** – Exsanguinate the rabbits.

#### B. Preparation of Conjugates

##### 1. Specific LDB conjugates

Specific antisera for the four known serogroups of LDB were raised in rabbits by immunizing with cells of the representative strains according to the above schedule. Sera were fractionated and labeled with fluorescein isothiocyanate according to the methods of Hebert et al. (7). Serogroup 1 comprises strains of LDB, such as Knoxville 1, that are stained brightly (3+ to 4+ intensity) with relatively high dilutions of the Knoxville 1 conjugate, and others, such as the Bellingham 1 strain, that are only stained to a 3+ to 4+ intensity when relatively low dilutions of the Knoxville 1 conjugate are used. Thus, serogroup 1 conjugates must be titrated against both types of the group 1 strains to insure that a sufficiently low working dilution is used to detect all serogroup 1 LDB organisms. A polyvalent conjugate was prepared by combining appropriate volumes of the four serogroup-specific conjugates so as to give 3+ to 4+ staining of all of the available isolates of the four known serogroups of LDB. The number of serogroup-specific conjugates that can be practically included in a polyvalent preparation is limited because the total fluorescein concentration is higher in a polyvalent reagent. Eventually, as the number of group specific conjugates is increased, nonspecific or background fluorescence of the stained tissue specimen becomes a problem. Thus, if additional serogroups of LDB are found it will probably be necessary to use two polyvalent conjugates for diagnostic staining.



## 2. Counterstain (rhodamine-labeled rabbit serum)

Deparaffinized tissue sections and sputum smears are difficult to examine for LDB by the direct FA test because of the bright background formed by the nonspecific uptake of fluorescein-labeled globulin. Whole normal rabbit serum labeled with tetramethylrhodamine isothiocyanate has been found to be useful as a counterstain with these types of specimens.

### a. Materials

1. Whole serum from nonimmunized rabbits.
2. Tetramethylrhodamine isothiocyanate (TMR1). This reagent can be purchased in 100-mg lots from Baltimore Biological Laboratories, Baltimore, Maryland, or from Research Organics, Inc., Cleveland, Ohio.
3. Phosphate buffered saline (PBS) – 0.5 M sodium phosphate, pH 7.6, containing 0.85% NaCl and 0.1%  $\text{NaN}_3$ .
4. Dimethylformamide (DMF).
5. 0.5 M  $\text{Na}_3\text{PO}_4$ .
6. Sephadex G-25 fine (Pharmacia Fine Chemicals, Piscataway, New Jersey).

### b. Procedures

A 40-ml aliquot of whole serum is adjusted to pH 9.5 by adding 0.5 M  $\text{Na}_3\text{PO}_4$ . A 100-mg portion of TMR1 is dissolved in 2 ml of DMF before being mixed with serum. The reaction mixture is allowed to stand for at least 1 h at room temperature and then centrifuged to remove particulate matter. Unreacted rhodamine products are removed by gel filtration on a Sephadex column. A column 5 cm in diameter by 30 cm in length packed with 100 g of Sephadex G-25 fine that has been presoaked in PBS is adequate for this purpose. The first colored fraction to emerge from the column is the rhodamine-labeled serum. This fraction is followed by an orange fraction and a red fraction which are discarded. The column can be washed fairly clean of rhodamine products and used repeatedly. The whole serum-rhodamine conjugate should be dialyzed against PBS to remove traces of DMF, adjusted to a 160-ml volume by adding PBS, and filtered through a 0.45  $\mu\text{m}$  filter.

### c. Application

The anti-LDB conjugate, at four times the usual working concentration, is combined with three parts of the counterstain. This mixture is used in the usual manner for FA staining of LDB in tissue sections or in sputum specimens. If the anti-LDB conjugate is in the lyophilized state, it is rehydrated by adding the appropriate volume of counterstain to produce the desired working concentration. When a counterstain is used with the diagnostic conjugate, a similarly prepared control conjugate must also be used.



## II. LIST OF LDB ISOLATES

- A. Serogroup 1 isolates of the Knoxville 1 type include:
- |                    |                       |
|--------------------|-----------------------|
| Knoxville 1        | Pontiac 1 (E)*        |
| Philadelphia 1-4   | Miami Beach 1         |
| Flint 1 and 2      | Orlando 1             |
| Detroit 1-4        | Indianapolis 1 and 2  |
| Berkeley 1         | Oak Lawn 1            |
| Birmingham 1       | Baltimore 1           |
| Burlington 1 and 2 | Houston 1             |
| Buffalo 1          | Chicago 1             |
| Allentown 1        | Long Beach 1 and 2    |
| Albuquerque 1      | Cambridge 1 (England) |
| Los Angeles 2-14   | Atlanta 3             |
| New York 1 and 2   | Bellingham 1          |
| Olda               | Gastonia 1            |
| Rochester 1        | TOTAL = 50            |
- B. Serogroup 2 isolates obtained to date include:
- Togus 1
- Atlanta 1 and 2
- C. Serogroup 3 isolates obtained to date include the environmental representative Bloomington 2 (E). The human isolates Chicago 2 and 3 and Houston 2 also stain with serogroup 3 conjugate.
- D. Serogroup 4 is represented by the Los Angeles 1 strain obtained from human tissue.

All strains listed above were obtained from human patients with LD except Bloomington 2, an environmental isolate which is the type strain of serogroup 3, and Pontiac 1, which was isolated from a guinea pig naturally infected by the aerosol route. Thus 49 or 87.5% of the 56 human isolates listed above belong to serogroup 1. A number of other environmental isolates not included in the above list have been obtained that belong to serogroups 1-4. Still other environmental isolates have been found that apparently represent new serogroups but these cultures have not been completely characterized.

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\* — Environmental isolate

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# Demonstration of the Bacterium in Tissue by a Modification of the Dieterle Silver Impregnation Stain

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# Demonstration of the Bacterium in Tissue by a Modification of the Dieterle Silver Impregnation Stain

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The modification of the Dieterle silver impregnation stain described below is our adaptation of the staining procedure which Dr. Robert Dieterle developed for demonstrating *Spirochaeta pallida* in single microscopic sections. We modified his procedure to meet our need for a batch staining method and have used it successfully for a number of years. Recently, interest in this modified method increased after we used it successfully to demonstrate the Legionnaires' disease bacterium (LDB) in paraffin-embedded tissue sections. The routine staining procedures for bacteria and fungi were not satisfactory; therefore, the modified Dieterle staining procedure is a valuable diagnostic tool. Instructions for the procedure are detailed below.

## TISSUE AND SLIDE PREPARATION

Fix lung tissue for at least 24 hours in 20 times its volume of 10% buffered Formalin. Trim the tissue with a sharp blade to not more than 3 x 1.5 x 0.5 cm. Process according to the following schedule:

1. Place in 70% alcohol (two changes of 1 hour each).
2. Transfer to 95% alcohol (two changes of 1 hour each).
3. Transfer to absolute alcohol (two changes of 1 hour each).
4. Place in equal parts of absolute alcohol and chloroform for 1 hour.
5. Transfer to chloroform (two changes of 1 hour each).
6. Place in melted paraffin (two changes of 2 hours each).
7. Place tissues in a third change of melted paraffin until they are embedded.
8. Transfer tissue to an embedding mold and fill the mold with fresh melted paraffin. Allow the paraffin to harden, remove the paraffin block from the mold, and trim its face of excess paraffin.
9. Cut thin sections (4-6 micrometers) from the paraffin-embedded tissue blocks and float the sections on warm water in a floatation water bath. If a pinch of gelatin was dissolved in the warm water, pick up a floating tissue section with a plain glass slide. When gelatin was not used, pre-coat the glass slides with egg albumin before picking up sections. (NOTE: Albumin on the slide surface may be a source of nonspecifically staining artifacts.)

## STAINING PROCEDURE

Glass or plastic staining racks must be used or the staining can be done in coplin jars. Metal containers must not be used because the staining reagents react with metals to form a black precipitate which adheres to the tissue sections. All glassware must be thoroughly cleaned. Include a known positive control in each staining rack or coplin jar.

1. Remove the paraffin and hydrate the tissue sections by sequentially dipping the slides in two changes each of xylene, absolute alcohol, 95% alcohol, and distilled water.
2. Place slides in preheated 5% alcoholic uranyl nitrate at 55°- 58°C for 1 hour.
3. Dip slides in distilled water.
4. Dip slides in 95% alcohol.
5. Place slides in 10% alcoholic gum mastic for 3 minutes.
6. Dip slides quickly in 95% alcohol.
7. Place slides in distilled water for 1 minute.
8. Allow slides to drain for 15-20 minutes. (Slides can be left to dry overnight and the procedure completed the following day.)
9. Place slides in preheated 1% silver nitrate solution in 55°- 58°C oven in the dark for at least 4 hours. (The silver will precipitate if the oven temperature exceeds 60°C.)
10. Dip slides twice quickly in distilled water.
11. Dip slides in developing solution until sections are yellow to tan—usually in 1-2 minutes (check control slide microscopically for proper development of organisms).
12. Dip slides twice in distilled water.
13. Dip slides twice in 95% alcohol.
14. Dip slides twice in acetone.
15. Dip slides twice in xylene.
16. Place slides in a second dish of xylene.
17. Coverslip as soon as possible with Permunt or Protex mounting medium.

## INTERPRETATION

Scan the sections on a microscope with the low power objective. Locate areas of consolidation in which the alveoli are filled with fibrin and leukocytes. In most cases, we have found more organisms in the field containing the most degenerated leukocytes.

Under the high dry objective, the LDB appears as a 0.4-0.8 micrometer by 2-4 micrometer dark-brown to black rod against a yellow to tan background. Although these organisms may be intracellular or extracellular, most are found within macrophages. Spirochetes and some other bacteria also stain dark-brown to black. In paraffin-embedded tissue sections, bacteria which stain dark-brown to black with this procedure and have the morphological characteristics of the LDB but do not stain with tissue Gram stains can be presumed to be the LDB (Figure 1).

Other structures including melanin granules, chromatin, Formalin pigment, and some foreign material in macrophages may also stain by this method, but they can be readily distinguished from the LDB.

## DISCUSSION

The modified Dieterle silver impregnation stain has also been successfully applied to destained sections previously stained by hematoxylin and eosin or by tissue Gram stains. After removing the coverslips (by soaking slides in xylol) and destaining the sections in acid alcohol (by dipping until colorless), we have used the modified Dieterle procedure to demonstrate LDB in sections that have been filed for as long as one year.



We should mention that stained organisms may fade unpredictably. Although some of our slides have retained the stain for over a year, others have faded within hours—especially if they were not coverslipped immediately after staining.

We have had some success in preventing this fading by modifying the staining procedure as follows:

- (1) After the distilled water rinse in step 12, dip the slides in a 10% solution of 88% formic acid.
- (2) Rinse the slides once in distilled water and then proceed with step 13.

We have not been able to stain Zenkers'-fixed material successfully; other metal-containing fixatives may also adversely affect the staining of organisms.

To determine whether tissues subjected to decalcification solutions could be successfully stained, we used some tissue that had been treated with our routine decalcification solution (equal parts of 5% formic acid and 5% Formalin) and other tissue treated with a commercial decalcification solution (RDO from Dupage Kinetic Laboratories). Staining was not affected by either decalcification procedure.

As previously stated, a known Dieterle positive control slide should be stained in each rack of tissue sections. Control tissue for this procedure is available from the Center for Disease Control, Attention: Mrs. Pat Greer, MT (ASCP), Control Tissue Repository, Bureau of Laboratories, Atlanta, Georgia 30333.

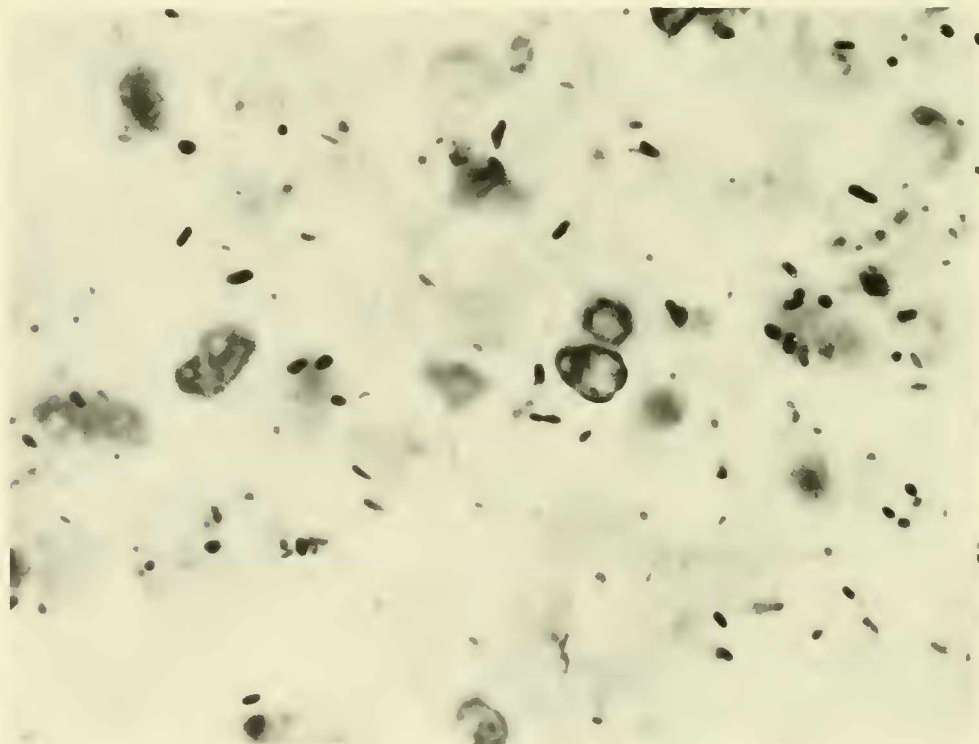


Figure 1. Legionnaires' disease bacteria in an area of consolidation. (Dieterle silver impregnation stain, X1500).

## REAGENTS

1. 5% alcoholic uranyl nitrate. Dissolve 50 gm uranyl nitrate in 1,000 ml of 70% alcohol. Stable in refrigerator for months.

2. 10% alcoholic gum mastic. Mix 100 gm gum mastic in 1,000 ml absolute alcohol, and allow 2-3 days to dissolve. Filter solution and store in refrigerator in a well-stoppered bottle. Gum mastic is available from O.G. Innes Corp., 10 East 40th Street, New York, NY 10016.

3. 1% silver nitrate. Dissolve 10 gm silver nitrate in 1,000 ml of distilled water. Store in refrigerator. Discard solution if it turns dark.

4. Developing solution - Mix in order:

Hydroquinone	15.0 gm
Sodium sulfite	2.5 gm
Distilled water	600.0 ml
Acetone	100.0 ml
Formalin (37%-40%)	100.0 ml
Pyridine	100.0 ml
10% alcoholic gum mastic	100.0 ml

Swirl flask gently as each solution is added. Solution turns milky yellow as the gum mastic is added and light brown after standing in a well-lighted area. As brown streaks appear, swirl the flask gently. Developing solution is ready to use in about 6 hours and can be used until it turns dark brown (usually in 2-3 days).

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# Indirect Immunofluorescence Test for Legionnaires' Disease

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## Indirect Immunofluorescence Test for Legionnaires' Disease

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### INTRODUCTION

The indirect immunofluorescence (IF) test for Legionnaires' disease (LD) has been modified several times since it was first used by McDade et al. to provide serological evidence that an organism which had been isolated from patients with Legionnaires' disease was actually the causative agent of that disease. We now know that at least four different serogroups exist, with individuals responding immunologically in different ways to serogroup-specific antigenic determinants. Some patients appear to respond to the Legionnaires' disease bacterium (LDB) with a group-specific antibody response; others respond to antigenic determinants presumably common to serogroups 1-4. Preliminary data obtained at the Center for Disease Control (CDC) suggest that a polyvalent antigen (not yet in general use) can be used to screen sera for LDB antibodies. Positive sera are then titrated against monovalent antigens. We now use heat instead of diethyl ether to kill the organisms because ether extracts or destroys the serogroup 2 (Togus 1) antigen. Further modifications of the test may be necessary as we gain more knowledge about the organism and about the immune response to the LDB.

Perhaps because specific diagnostic criteria for LD have not been completely formulated, expectations of sensitivity, specificity, and reproducibility have been unusually high for the LD indirect IF test. Nevertheless, it is limited by the same immunologic parameters that affect other serological tests. For example, repeat tests in which titers are obtained that vary by no more than one 2-fold dilution are generally considered acceptable within limits of experimental error. Therefore, paired sera should be tested simultaneously. A 4-fold rise in titer from the acute to the convalescent phase of illness is usually considered the only solid serologic evidence of infection. Some sera from patients with LD do not contain detectable antibodies against the identified "infecting" strain of the LDB, whereas sera from some other patients infected with dissimilar pathogens may contain antibodies that react with LDB, presumable because of similarities of the heterologous organisms' antigenic structure or perhaps because of nonspecific stimulation of the reticuloendothelial lymphocytic system. It is interesting that most of the reported "cross-reactions" between LDB and other pathogens have not been confirmed. It would be unusual, however, for serologic cross-reactions not to occur with antigenic mimicry being so common in nature. No serologic test is 100% sensitive or specific. For these reasons, the indirect IF test should be used in conjunction with other diagnostic criteria.

The indirect IF test for LD has the following components: (a) an antigen composed of heat-killed, whole LDB cells, (b) appropriate dilutions of the human serum to be tested or of a control serum, (c) rabbit antihuman conjugate [fluorescein isothiocyanate (FITC)-labeled rabbit antibody with specificity for human immunoglobulins G and M], and (d) an appropriately equipped fluorescence microscope.

## PREPARATION OF BACTERIAL ANTIGENS

1. Inoculate the organism heavily on an agar slant of charcoal-yeast extract medium (20- X 150-mm screw-capped test tube containing 10 ml of medium). Incubate the loosely capped agar tube in a candle extinction jar at 35°C until good growth occurs (approximately 2 days).
2. Add 2 ml of sterile distilled water to the tube. Gently rub the growth off the slant with a Pasteur pipette, and transfer the suspension to a screw-capped test tube.
3. Place the tube containing the cell suspension in a boiling water bath for 15 min to kill the cells.
4. Streak an agar plate to confirm cell death. Use 0.1 ml of the cell suspension and the same medium and incubation conditions as in Step 1. Observe for growth for at least 10 days.
5. Pack the cells by centrifugation (2000 x g or about 5 min in a tabletop clinical centrifuge). Discard the supernatant fluid. Resuspend the bacterial sediment in 2 ml of sterile distilled water (concentrated antigen suspension).
6. Make working dilutions of the concentrated antigen suspension in 0.5% normal chicken yolk sacs (NYS) in sterile distilled water. (The procedure for preparation of NYS is detailed in the manual Appendix.) It is generally best to try several antigen dilutions in the 1:20 to 1:80 dilution range in the indirect IF test. High concentrations of bacterial cells can reduce titers, because proportionately fewer antibody molecules are bound per bacterial cell, and low concentrations make slide reading difficult because there are too few fields of cells and too few cells per field. Most antigens are dispersed optimally at a dilution of 1:50 (OD of 0.08-0.10 at 660 nm with 1-cm light path or approximately 500-600 organisms per microscopic field at a magnification of 315 X).
7. Add the preservative, sodium azide ( $\text{NaN}_3$ ), to the concentrated antigen and to the diluted "working" antigen to a final concentration of 0.05%.
8. Store antigens at 4°C. Although it is too early to know the results of long-term stability studies, antigens are apparently stable at 4°C for at least 2 mon.

## INDIRECT IMMUNOFLUORESCENCE TEST PROCEDURE

### A. Preparation of antigen slides

1. Thoroughly mix the working dilution of the heat-killed antigen suspension. Using a Pasteur pipette, apply enough antigen to each well of a microscope slide to cover the well with the antigen; remove the excess liquid with the same pipette (e.g., 25- X 75-mm acetone-resistant glass slide with 12 staggered wells 5 mm in diameter; Cel-Line Associates, Inc., Minotola, N.J.).
2. Allow the slide to air dry (may take up to 30 min).
3. Fix the antigen smears to the slide by placing the slide in an acetone bath for 15 min.
4. Allow the slide to air dry.
5. If slide is to be stored for future use, place in freezer at -20°C. Frozen slides are stable for at least 2 mon.

### B. Titration of Sera

1. Prepare a 1:16 dilution of the serum in 2.5%-3.0% normal chicken yolk sac (NYS)



suspended in 0.01 M phosphate buffered saline, pH 7.6 (PBS). Make subsequent doubling dilutions through 1:2048 in plain PBS. (We use Linbro Scientific Co. rigid polystyrene U-bottomed microtitration trays and a Cooke Engineering Co. automatic diluter. We place 0.01 ml of serum in a test tube containing 0.15 ml of NYS suspension and make subsequent doubling dilutions in wells of the microtitration tray using plain PBS and 0.05-ml volumes. Alternatively, replace the automatic diluter with hand diluters, or make all dilutions in test tubes.)

2. Using a Pasteur pipette, place a drop of each serum dilution from 1:64 to 1:2048 (or 1:256 if sera are being screened first) in its well of fixed antigen on a slide. Incubate the slide in a moist chamber (e.g., petri dish containing moist filter paper) at 37°C for 30 min.

3. Rinse slide quickly with PBS and place in a PBS bath for 10 min. Remove from the bath and gently blot dry.

4. Place a drop (approximately 20  $\mu$ l) of FITC-labeled antihuman immunoglobulin on each well. Incubate as above for 30 min.

5. Rinse slide quickly with PBS, place in a PBS bath for 10 min, rinse quickly with water, and gently blot dry.

6. Add several drops of buffered glycerol (1 part 0.5 M carbonate-bicarbonate buffer, pH 9, in 9 parts glycerol) to the slide and mount with a 24- X 60-mm coverslip (No. 1). (Check the pH of the buffered glycerol mounting fluid frequently; the fluid should not be used if below pH 8.)

7. Read slides on a fluorescence microscope (see below).

8. The serum titration end point is the highest dilution of serum that gives 1+ fluorescence (see below) of at least 50% of the bacteria estimated per microscopic field. The titer is the end-point dilution factor.

### C. Controls

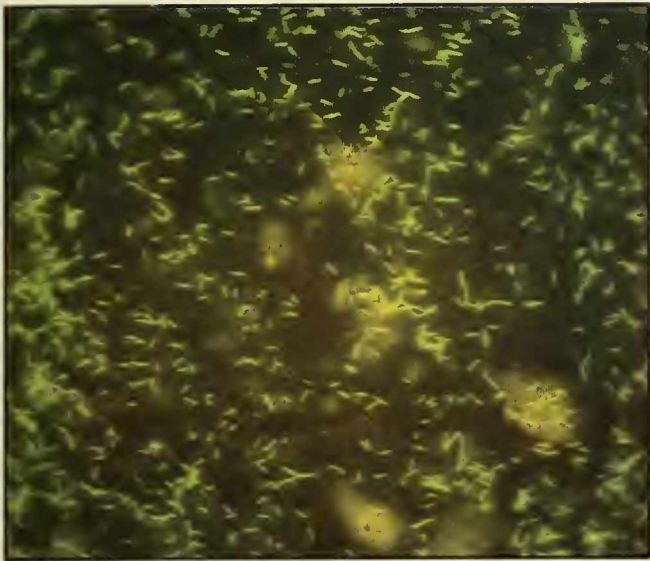
1. The primary control is a human serum having a specified titer against the serogroup antigen used. For the test to be considered valid, the titer obtained with the control serum should not vary more than 2-fold from the titer specified for that serum.

2. The second control is PBS substituted for serum, to test for nonspecific binding of the conjugate. If bacterial cells are visible, fluorescence intensity must be <1+ (see below).

---

### Legend for color plate:

Indirect IF test of patient's serum for antibodies against Legionnaires' disease bacterium, serogroup 1 (Philadelphia 1 strain). Dilutions of serum and resulting fluorescence intensities are given under each photograph. Staining endpoint = 1+. Titer = endpoint dilution factor = 512. Magnification = 315X. Note that autofluorescence (yellow-brown) of organisms at dilution of 1:1024 must not be interpreted as positive FITC fluorescence (green) and that FITC fluorescence must be read only for nonfilamentous, isolated cells in an area relatively free of clumps of fluorescent yolk sacs.



1:64 4+



1:128 3+



1:256 2+



1:512 1+



1:1024

#### D. Fluorescence Microscopy

1. Fluorescence intensities are read as follows:

4+ = brilliant yellow-green staining of bacterial cells

3+ = bright yellow-green staining

2+ = definite but dim staining

1+ = barely visible staining

– = no staining, although dim autofluorescence of the cells may occur with some filter systems (see below)

CAUTION: Disregard staining of filamentous cells and of cells on or adjacent to fluorescent yolk sac material.

2. Microscope Assemblies

We have found that the following give satisfactory results: Leitz Dialux 20 fluorescence microscope equipped with an HB0-100 mercury incident light source; the Leitz I-cube filter system (2 x KP490 and a 1-mm GG455 primary filter, TK510 dichroic beam-splitting mirror and K515 secondary filter) or Leitz K-cube filter system (same as I-cube except 1-mm GG455 filter is replaced by a 2-mm GG475 or a K480 edge filter to reduce background fluorescence), 40 X dry objective, and 6.3 X eyepiece (magnification- 315 X). Other microscope assemblies may also be satisfactory.

#### E. Interpretation\*

1. A 4-fold or greater increase in titer to  $\geq 128$  in sera obtained in the acute and convalescent phases of illness provides serological evidence of Legionnaires' disease. Optimal time intervals from onset of illness to specimen collection appear to be  $\leq 7$  days for the acute- and  $\geq 21$  days (but  $< 6$  weeks) for the convalescent-phase serum.

2. A single or standing titer of  $\geq 256$  provides presumptive evidence of an LDB infection at an undetermined time. Until more data are accumulated to show (a) how long a detectable level of antibody usually persists after infection with the LDB, and (b) the average LDB antibody concentration in sera of apparently normal individuals, the significance of a single high titer will remain unknown.

3. All other findings are considered negative.

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\*Although these interpretations appear to be valid for serogroup 1 infections, they may not be valid for those caused by members of other serogroups. The serogroup to which the infecting strain belongs cannot be determined unequivocally by results of the indirect IF test alone.



# Analysis of Cellular Fatty Acids of Bacteria by Gas-Liquid Chromatography

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# Analysis of Cellular Fatty Acids of Bacteria by Gas-Liquid Chromatography

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## INTRODUCTION

Chemical analyses of cell structure of bacteria and of products from their metabolism provide useful additional information for identification and classification. Extensive studies with cellular fatty acids have shown that certain closely related species can be distinguished on the basis of qualitative differences in their fatty acids (1, 2). A recent example is the Legionnaires' disease bacterium (LDB), which is different from other gram-negative bacteria because it contains large amounts (> 77%) of branched-chain fatty acids (5).

The lipids of bacteria are found in the cell wall/cell membrane fraction, where the unit fatty acids are chemically bonded to other cellular components. The acids are liberated or cleaved from these components and subsequently analyzed by gas-liquid chromatography (GLC). GLC is the method of choice for fatty acids because of its speed, sensitivity, and excellent separating efficiency. The following is a protocol for analysis of bacterial cellular fatty acids by GLC techniques with special reference to the LDB. A detailed review on GLC and its application to microbiology appeared recently (2).

## PREPARATION OF SAMPLES

**A. Saponification** -- to determine "total" cellular fatty acids, bound lipids must be freed by a saponification or hydrolysis procedure.

1. Add 0.5 ml sterile distilled water to the surface of an agar slant (16 x 150 mm).
2. Gently scrape the cells off the agar, and then transfer the turbid cell suspension to an 18 or 20 x 150 mm test tube with a Teflon-lined screw cap.
3. Add 4 ml of 5% NaOH in 50% aqueous methanol, and tighten the Teflon-lined cap.
4. Heat the cell suspension for 30 min in a boiling water bath (100°C).
5. Remove the tube from the water bath and cool to room temperature. If necessary, tubes may be held overnight in a refrigerator.

**B. Methylation** -- before conversion to esters, the sodium salts of the acids from saponification must be converted to the free form.

1. Add approximately 1.0 ml of 6 N HCl to the cool saponificate to lower the pH to 2.0.
2. Mix well and check with pH indicator paper. If needed, add more 6 N HCl drop by drop until pH is 2.0.
3. Add 4 ml of 10% boron trichloride-methanol reagent (Applied Science Laboratories, State College, PA) and mix well. Tighten Teflon-lined cap.
4. Heat for 5 min in a water bath maintained at 80°C-85°C.

5. Remove tubes from the water bath and cool to room temperature prior to extraction. For additional information regarding other esterification procedures see reference 4.

### C. Extraction of Methyl Esters

1. Add 10 ml of a 1:1 mixture of ethyl ether:hexane to the tube and tighten cap. Mix well, i.e., invert the tube 10-20 times and shake firmly for about 10 sec.
2. Allow the phases or layers to separate. Transfer the ether:hexane phase (top layer) to a 50- or 100-ml beaker. CAUTION: Avoid transferring aqueous phase along with the ether:hexane phase.
3. Add another 10 ml of ether:hexane to the aqueous phase (bottom layer) and repeat the extraction procedure of Step C-1.
4. Allow the phases to separate, and combine the ether:hexane layer with the first fraction already in the beaker.
5. Place the beaker under a gentle stream of flowing nitrogen gas in a chemical hood, and evaporate to reduce the combined 20 ml of solvent to approximately 0.5 ml. CAUTION: Never allow the sample to go to complete dryness during evaporation of solvent.
6. Add a small amount (80-100 mg) of  $\text{Na}_2\text{SO}_4$  to the beaker to remove residual moisture, and then transfer the sample to a 13- x 100-mm screw-capped tube. Rinse the beaker with a small volume of hexane, and then add the rinse to the sample tube.
7. Reduce the final volume (sample plus solvent) in the test tube to approximately 0.1 ml under a gentle flow of nitrogen gas in the hood. The methyl ester sample is then analyzed by GLC or is stored at  $-20^\circ\text{C}$ .

## GAS-LIQUID CHROMATOGRAPHY

### A. Equipment

The gas chromatographs used in this laboratory for cellular fatty acid analysis are equipped with flame-ionization detectors (FID) and temperature programs. Glass rather than metal columns is used to avoid possible destruction of hydroxy acids (2). A non-polar stationary-phase material such as the methyl silicones (SE-30, OV-1, OV-101) is used as the principal analytical column. These materials have excellent separating efficiency, temperature stability, and low column bleed, and are readily available from several commercial sources. A 3% concentration of one of these phases coated on an inert support (i.e., 100-120 mesh Gas-Chrom O) is packed into a 0.16 in (4.06 mm I.D.) x 12 ft (3.66 m) glass column. The column is placed into the instrument **without** connecting it to the detector and "conditioned" overnight at  $290^\circ\text{C}$  with a low carrier gas flow (20 cc/min) passing through the column. After conditioning, the column is connected to the detector, checked for leaks, and temperature programmed from  $100^\circ\text{C}$ - $270^\circ\text{C}$  several times prior to sample analysis. The carrier gas is adjusted to about 60 cc/min, and the instrument is set as follows: initial temperature,  $160^\circ\text{C}$ ; final temperature,  $270^\circ\text{C}$ ; temperature program rate,  $5^\circ\text{C}/\text{min}$ . Under these conditions, excellent separation of a fatty acid methyl ester mixture containing up to 23 components can be accomplished within 30 minutes.

### B. Column Control

A measure of the efficiency of column separation on non-polar phases is base line or near base line resolution of the methyl esters of palmitoleic (16:1) and palmitic (16:0) acids. Appropriate adjustments of carrier gas flow, initial column temperature, and temperature program rates



should be made to insure satisfactory separation of these two compounds. If these two components are resolved on a non-polar phase material, other esters which are present in a bacterial fatty acid sample will, in general, also be well resolved.

### C. Procedure

For analysis, 1-3  $\mu$ l of the bacterial fatty acid methyl ester sample is injected into the column, and a tracing (chromatogram) of the separated components of the mixture is recorded on a strip chart. The retention time (time from injection to the center of the eluted peak) of each peak is recorded and compared to retention times of those in a reference standard. The presence of specific fatty acids and their relative concentration compared to those of other acids in the sample are the bases for differentiation (2).

## IDENTIFICATION

A significant amount of information on the identity of the fatty acids in the bacterial sample can be obtained by comparing GLC retention data to highly purified reference standards on both non-polar (i.e., OV-101) and polar [i.e., ethylene-glycol-adipate (EGA)] stationary-phase columns. On non-polar phases, fatty acid methyl esters are separated by boiling points. Thus, unsaturated acids elute before their saturated homologs, saturated branched-chain acids before their saturated straight-chain homologs, cyclopropane acids before their straight-chain homologs, and 2-hydroxy acids before their 3-hydroxy homologs. On polar columns such as EGA, the elution sequence is reversed for saturated acids and their unsaturated homologs. Iso- and anteiso-isomers of saturated branched-chain acids also separate to some degree on polar columns (3). Thus identical retention time matches on both polar and non-polar phase materials give strong preliminary identification. In addition, the presence of unsaturated acids can be confirmed by bromination or hydrogenation. Treatment of the methyl ester sample with bromine results in the addition of this compound across the double bonds to produce a much less volatile species (bromo-methyl ester), which does not appear in the chromatogram under the initial conditions of GLC upon subsequent GLC analysis. Similarly, hydrogenation converts unsaturated esters to their corresponding saturated esters, which causes the GLC peaks representing esters of unsaturated acids to disappear and causes the peaks representing saturated acid esters to increase in size. The presence of a hydroxy acid can be confirmed by treating the methyl ester sample with an anhydride (e.g., acetic, trifluoroacetic) to form the diester derivative. Upon subsequent analysis by GLC, the peak representing the methyl ester derivative will disappear from the chromatogram, and a new peak representing the diester derivative will appear. The diester will elute from the column more quickly (shorter retention time) as a result of its increased volatility.

### A. Hydrogenation of Methyl Esters

1. After analysis by GLC, reduce the methyl ester sample almost to dryness under nitrogen.
2. Add approximately 0.5 ml of a 3:1 mixture of chloroform:methanol down the side of the tube and mix well.
3. Transfer the sample to a 10-ml vacuum hydrolysis tube (Kontes Glass Company).
4. Add a small amount (5 mg) of catalyst (5% platinum on charcoal) and a small, Teflon-coated stirring bar.
5. Connect the tube to a ring stand and suspend the tube over a magnetic stirrer. Close the upper part of the tube and mix the contents by stirring.

6. Turn off the stirrer, open the stopcock in the upper part of the tube, and bubble a small volume of hydrogen gas into the tube for approximately 5 sec. CAUTION: Hydrogen is a highly flammable gas and must be handled with extreme caution.
7. Close the stopcock and mix the contents for 15 min with the magnetic stirrer. Add a small volume of hydrogen gas two (more) times, and allow 15-min intervals for stirring.
8. Remove the catalyst by filtration through a small Whatman paper filter (#1) and collect the hydrogenated sample in a 13- x 100-mm screw-capped tube.
9. Reduce the sample volume to about 0.1 ml under a gentle flow of nitrogen gas in the hood.
10. Analyze 1-3  $\mu$ l of sample by GLC under identical conditions to those described for the original methyl ester sample.

#### B. Acetylation of Methyl Ester

1. After analysis by GLC, add 5 drops of trifluoroacetic anhydride to the methyl ester sample tube with a Pasteur pipette.
2. Mix gently and leave at room temperature for 30 min.
3. Add 0.3 ml distilled water and mix gently to wash the sample.
4. Allow the layers to separate. Carefully transfer the hexane layer (top) to a clean 13- x 100-mm screw-capped tube.
5. Analyze 1-3  $\mu$ l of the acetylated sample by GLC under identical conditions to those described for the original methyl ester sample.

### QUANTITATION

As noted previously, the bases for differentiation among bacteria are the presence of specific fatty acids and their relative concentration compared to those of other acids in the sample. Therefore, in addition to observing the overall general fatty acid profile, it is desirable to determine the relative concentration of peaks in the chromatogram. Peak areas are obtained from the chromatogram (DISC or electronic integrator), and the percentage of each acid is calculated from the ratio of the area of its peak to the total area of all peaks. In this laboratory, small peaks with areas less than 2% of the total are disregarded.

### CONTROLS

Because of the extreme sensitivity of the technique, highly purified chemicals, solvents, reagents, and gases are used in GLC studies. Glassware is a potential source of contamination and should be checked for cleanness and cracks. Teflon-lined caps should be used, and these and all glassware should be rinsed with solvents (hexane, acetone) before use. A bacterium of known fatty acid composition should be included along with the unknown organism. Also, a "reagent blank" (i.e., distilled water) should be processed occasionally as a check on all reagents, solvents, and the GLC column.

## SUMMARY OF GLC ANALYSIS OF BACTERIAL CELLULAR FATTY ACIDS

- A. **Saponification** — 4 ml of 5% NaOH in 50% methanol is added to cells from a slant culture and heated for 30 min at 100°C. The sample is cooled.
- B. **Methylation** — The pH is adjusted to 2.0 with 6 N HCl; 4 ml of 10% boron trichloride-methanol reagent is added, and the mixture is heated at 80°C for 5 min. Sample is cooled.
- C. **Extraction** — Methyl esters extracted from mixture with two 10-ml aliquots of a 1:1 mixture of diethyl ether:hexane. Solvent containing methyl esters is reduced to about 0.1 ml.
- D. **GLC** — 1-3  $\mu$ l of methyl ester sample is injected into non-polar GLC column and analyzed. Retention times of peaks are recorded and compared to reference standards. Quantitative data are obtained, and identities of fatty acids are confirmed by additional procedures (hydrogenation, acetylation, analysis on polar column, mass spectrometry).

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# **ELISA for Legionnaires' Disease - Antibody and Antigen Detection Systems: An Interim Report**

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# ELISA for Legionnaires' Disease - Antibody and Antigen Detection Systems: An Interim Report

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## INTRODUCTION

In many cases, laboratory confirmation of Legionnaires' disease (LD) is accomplished retrospectively either by indirect immunofluorescence (IF) tests of acute- and convalescent-phase sera (7), or by direct IF tests of postmortem lung tissue (2). Previously we reported a micro enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against serogroup 1 LDB soluble antigens (3), using the horseradish peroxidase enzyme. Although the test appears promising, its sensitivity and specificity need further refinement and evaluation. The primary disadvantage of using antibody detection tests is the delay involved in seroconversion (approximately 3 weeks) and, therefore, in diagnosis.

Earlier diagnosis of LD is needed so that specific therapy can be instituted during the acute stage of illness. Accordingly, we have investigated the use of ELISA for the detection of serogroup 1 antigen in urine (1).

In a preliminary evaluation of the ELISA, antigen was detected in the urine of all six guinea pigs tested within 4 h and for at least 1 wk after they were inoculated with nonviable LDB serogroup 1 (Philadelphia 1 and 2 and Knoxville 1). Urine specimens from animals inoculated with the serogroup 2 strain (Togus 1) were negative in the serogroup 1 ELISA, as would be expected because of the antigenic dissimilarity of these serogroups (5, 7). Urine specimens from control animals were negative.

Urine specimens from 20 human subjects with no recent history of respiratory disease were also negative. Antigen was detected, however, in urine which had been collected in 1976 from three of four LD patients during the Philadelphia outbreak (4). Urine from a fifth patient with a clinically compatible illness but equivocal serological test results was negative in the ELISA. Since this work was completed, investigators conducting an independent limited study have used ELISA to detect antigen in sputum and urine from two LD patients (6).

## ELISA FOR ANTIBODY

The micro-ELISA for detecting antibodies to the Legionnaires' disease bacterium (LDB) involves a soluble antigen prepared from heat-killed organisms. Comparison testing of this procedure and the indirect IF test has not been completed. ELISA appears to be a promising laboratory tool which continues to be improved with research on other enzyme systems and immunopurified conjugates. The instructions for performing the micro-ELISA for antibody titration are shown below:

### A. Antigen Preparation

1. Grow the LDB on Mueller-Hinton agar plates supplemented with 1% IsoVitaleX (BBL) and 1% hemoglobin for 72 h at 37° C in a candle jar.

2. Working in a microbiological safety hood, harvest growth by adding 3 ml of sterile phosphate buffered saline (PBS), pH 7.2, to each agar plate and gently scrapping the agar with a bent capillary pipette.
3. Transfer the cell suspension to a sterile screw-capped flask, and steam in an Arnold sterilizer for 1 h at 101°C.
4. Centrifuge the killed-cell suspension in sterile 15-ml conical centrifuge tubes for 30 min at 1600 X g. Decant the supernatant, and wash packed cells twice in sterile PBS, pH 7.2.
5. Prepare a stock suspension of the cells by adding 2 ml of sterile PBS, pH 7.2, to each 0.1 ml of packed, centrifuged cells. Store stock antigen suspension at 4°C for 10 days to allow the release of soluble antigen from the cells.
6. Centrifuge the suspension at 1600 X g. Save the supernatant to be used as the stock soluble antigen. (By doing block titrations, we found that a 1:40 dilution was adequate with our system.)

## B. Test Procedure

1. Dispense 0.1-ml volumes of diluted antigen into each well of as many Cooke micro-ELISA plates as are needed (protein-binding polystyrene no. 1-223-29). Seal plate with Cooke Microtiter plate sealers, incubate at 37°C for 3 h, and store at 4°C until used.
2. Aspirate excess antigen from wells, and wash each plate at least three times with PBS, pH 7.2, containing 0.05% Tween 20. Leave PBS in the plate for at least 3-4 min per wash.
3. If numerous sera are to be tested, screen them at a 1:8 or 1:16 dilution. Set up two wells per specimen.
4. Determine the end points of the positive sera by making further 2-fold dilutions. Assign each specimen to be tested a row of wells on a plate. Also assign one row to a known negative serum and one row to a known positive serum for each test run. Include PBS control.
5. With an automatic pipette, dispense 0.1 ml of a 1:8 or 1:16 dilution of the specimen to be tested into well 1 of the row assigned to it. Prepare serial 2-fold dilutions as follows. To wells 2 through 8 assigned to a specimen, with a Cooke microdropper add 0.05 ml of PBS, pH 7.2, containing 0.05% Tween 20. Then, make 2-fold dilutions with Cooke microdiluters (0.05 ml). (See Figure 1.) Finally, add 0.05 ml of PBS, pH 7.2, with 0.05% Tween 20, to all wells on each plate, and vibrate or gently mix.
6. Incubate plates at 37°C for 1 h.
7. After they are incubated, wash plates as in Step 2.
8. Predetermine appropriate conjugate dilution by block titration. Dispense 0.1 ml of the appropriate dilution of enzyme-labeled conjugate [horseradish peroxidase(HRPO)-labeled, antihuman immunoglobulin] into each well of every plate.  
NOTE: Assaying and adding the conjugate are perhaps the most critical steps in the procedure. Impure and nonspecific conjugates adversely affect both specificity and sensitivity.
9. Cover plates, and incubate them at 37°C for 1 h.
10. Wash as described in Step 2 using five washes.
11. Add 0.1 ml substrate to each well. The substrate for HRPO-labeled conjugate is ortho-phenylenediamine (OPD). Stock contains 1% (wt/vol) OPD in methanol (100 mg OPD/10



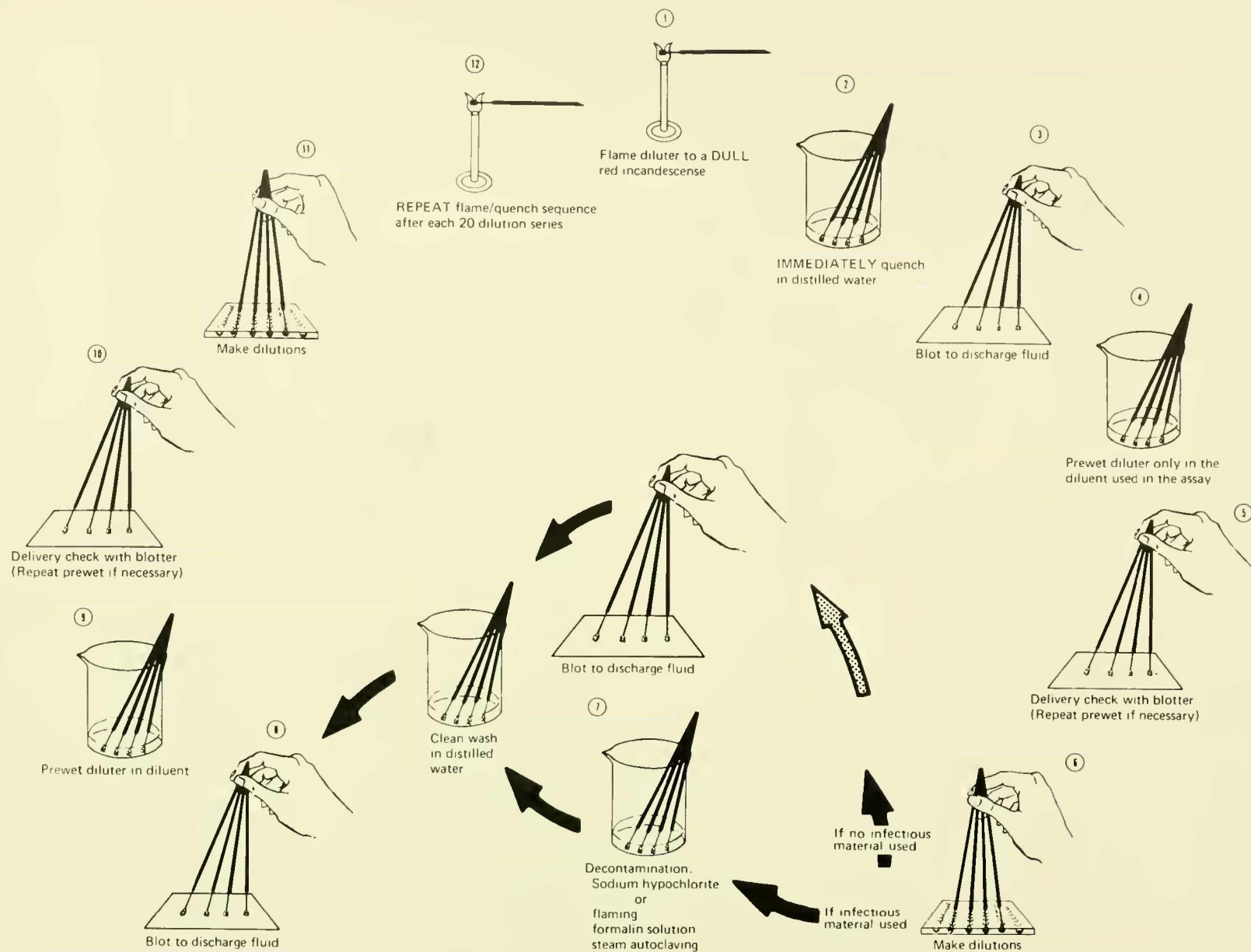


Figure 1. Microtitration Diluter Cycle

ml ethanol). Stock does not deteriorate if stored in the dark for up to 1 wk. Working substrate is composed of 1 ml of stock plus 99 ml of distilled water containing 0.1 ml of 3% hydrogen peroxide.

12. Cover plates and incubate in the dark at 37° C for 30 min to 1 h, depending upon the color reaction desired.

13. Remove plates from the incubator; add 1 drop of 8 N H<sub>2</sub>SO<sub>4</sub> to each well to terminate the reaction and deepen the color.

### C. Interpretation

Place the plate on a white background or on a reading mirror, and grade the color of each well against that of the partially colorless negative control. The end point is defined as the highest dilution of serum which is distinctly different in color from the first dilution of the negative control. Report titer as the reciprocal of the end point.

### D. Limitations

Because the sensitivity and specificity of this developmental procedure are not adequately defined, sera for which **positive** or **doubtful** or **equivocal** results are obtained should be referred to the state health department for confirmatory testing with the indirect IF procedure.

## ELISA FOR ANTIGEN

This ELISA is a "sandwich" technique with four layers (Figure 2) and uses the alkaline phosphatase enzyme detection system. The ELISA can detect antigen representing as little as 3 pg of protein in a purified lipopolysaccharide protein complex which is a major antigenic constituent of serogroup 1 LDB (8). Further antigen-detection studies with respiratory secretions and with acute-phase sera, as well as efforts to incorporate other serogroups into the test system, are clearly warranted. In addition, sensitivity and specificity must be determined. Until these parameters are defined a detailed procedure cannot be recommended.

## SUMMARY

Although still in the developmental stages, ELISA for both antibody and antigen detection appear to offer promise as aids in the diagnosis of Legionnaires' disease.

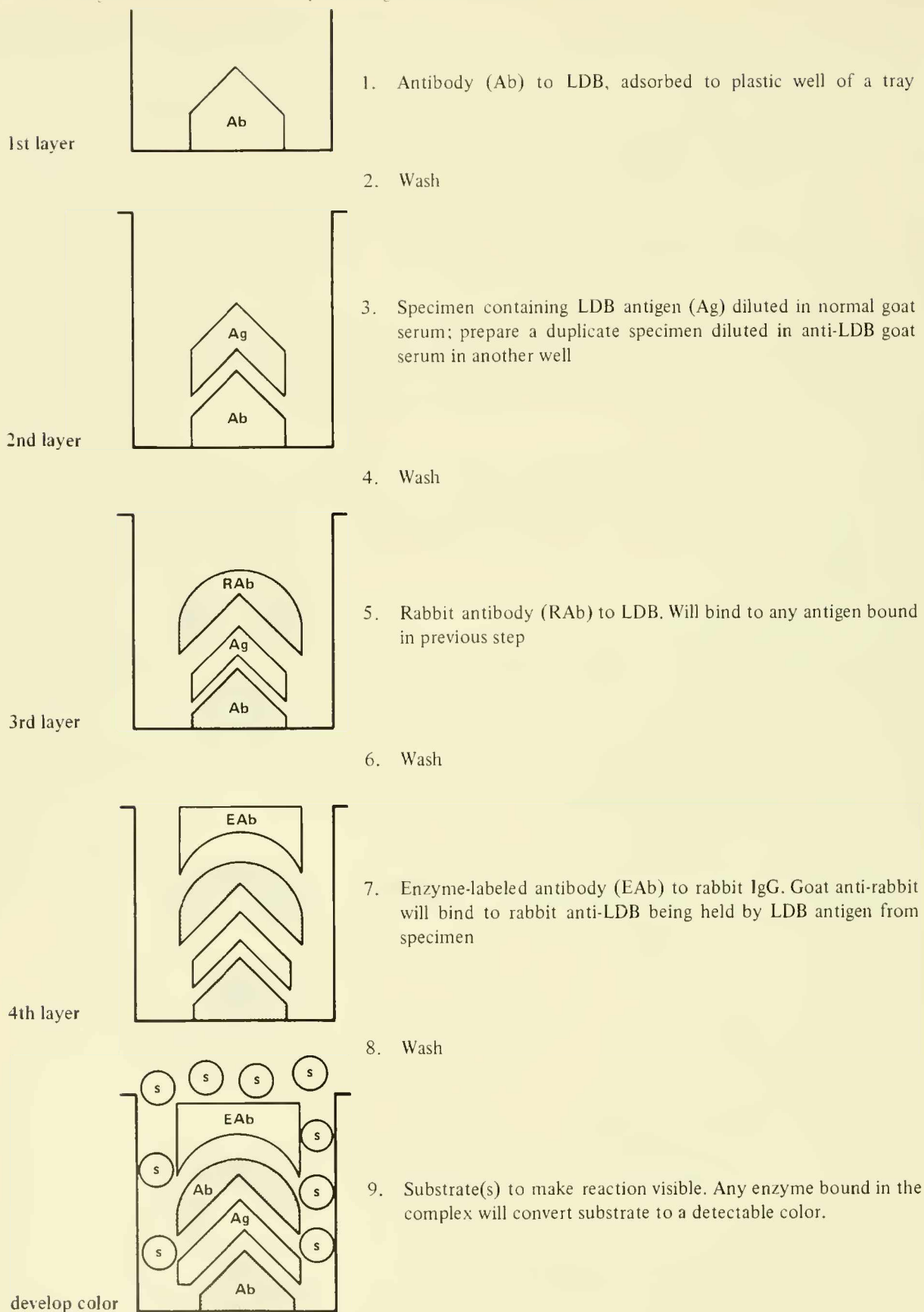


Figure 2. ELISA: 4-layer antigen detection system



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# **PART FOUR**

## **Policies, Resources and Services**





## Policies, Resources and Services

This section catalogues most aspects of the Center for Disease Control (CDC) assistance program pertaining to Legionnaires' disease (LD). Included are procedures to follow in obtaining (1) technical support and consultation and (2) diagnostic materials and services beyond the scope of your institution. The expertise of our laboratory staff during the adaptation and development of diagnostic methods is also available.

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## I. LABORATORY SAFETY: PRECAUTIONS AND PRACTICES

Apparently Legionnaires' disease is acquired by the airborne route, presumably after droplet nuclei containing the Legionnaires' disease bacterium (LDB) are inhaled. Laboratories must also consider a second route – the puncture of the skin with equipment or broken glass contaminated with the LDB. The following general precautions and laboratory practices are minimal safety standards for handling infectious material. In addition, most of the contributors to this manual included in their procedures or discussions safety measures that are pertinent to the techniques detailed in their chapters.

### A. Aerosols

Many laboratory manipulations generate potentially infectious aerosols; every effort should be made to minimize aerosol formation and to contain any such aerosols formed. Appropriate precautions can significantly reduce aerosols, which are generated whenever a liquid surface is disrupted, e.g., the liquid film between the cap and the wall of a container stretching and breaking when a screw cap or cover is removed; bubbles of fluid bursting at the tip of a pipette or on the surface of a fluid after vigorous shaking or mixing; fluids spattering from heated wire loops; wire loops vibrating after inadvertently touching the sides of tubes or containers; tubes breaking or leaking in a centrifuge; streaking agar media in tubes or plates, especially when medium surface is rough (resulting from bubbles in the medium when it was poured). The following practices help minimize and/or contain aerosols.

1. Always perform operations such as transferring cultures and preparing microscope smears for examination in a biological safety cabinet.
2. Perform any procedure which produces aerosols but cannot readily be done in a safety cabinet (such as centrifuging in a large centrifuge, shaking broth cultures during incubation, or using a high-speed blender) with safety equipment that will prevent the dissemination of infectious aerosols if the primary container breaks or leaks.
3. Streak agar plates which have a smooth instead of rough agar surface and thus reduce aerosol formation by 99%.
4. Insert a cool rather than hot inoculating loop into media. This will reduce aerosols 90%-95%.
5. Allow the last drop in the pipette to drain out instead of blowing it out. This will reduce aerosol formation 67%.
6. Mix cultures in a tube instead of a pipette and thus reduce droplet nuclei by 100%.
7. Work over a disinfectant-soaked gauze sheet (rather than on the hard surface of a bench top) to absorb falling drops of culture material and reduce aerosols by 90%.
8. Insert a needle wrapped with an alcohol pledget through the top of a stoppered vaccine bottle to release the pressure. This reduces aerosol production by 99%.
9. Leave screw caps on centrifuge tubes to allow contents to settle before opening caps, and reduce aerosols considerably.
10. Centrifuge in safety cups to eliminate essentially all aerosol formation in the event of tube breakage.



## B. Contaminated Materials

1. Perform any procedure that involves a potentially viable antigen very cautiously.
  - a. Fix impression smears of fresh tissue in 10% Formalin.
  - b. A heat-fixed smear may still contain a viable organism; therefore discard most stained microscope slides into a pan of disinfectant for autoclaving.
  - c. After staining, autoclave the rinse buffers and the towel onto which a direct immunofluorescence slide is drained.
2. Decontaminate work surfaces after processing or otherwise handling potentially infectious materials.
3. Immediately cover any spilled culture or specimen with Clorox® at 500 ppm. Disinfect counter tops in work areas with an Environmental Protection Agency-registered phenol-base disinfectant. If the culture contained a toxin, flood with 1 N NaOH to neutralize the toxin.
4. Autoclave all glassware, laboratory wastes, animal bedding cages, and other potentially contaminated items before discarding or recycling.

Place discard material in a leak-proof pan with a loosely fitting cover; add approximately 1 in of water to the pan and autoclave for 1 h. If bio-bags are used, fill them only half-way, add a small amount of water, and leave tops open for air exchange during autoclaving.

5. When an experimental animal is to be discarded, autoclave it before it is incinerated.

## C. Lyophilized Cultures

Most of the reference cultures obtained from the Center for Disease Control (CDC) are lyophilized and sealed under vacuum. Do not remove the top or release the vacuum before reconstituting them. **Perform work in a biological safety cabinet.**

1. Reconstitute the lyophilized culture by aseptically injecting 1.0 ml of sterile distilled water through the rubber stopper with a sterile needle and syringe.
2. Without removing the needle and syringe, swirl the vial to resuspend the contents. Invert the vial, and withdraw the cell suspension while holding a cotton pledget at the top of the vial to prevent an aerosol or puncture as the needle is withdrawn through the stopper.
3. Transfer the cell suspension to a small sterile test tube. (Insert a disposable needle through the top of the vial and autoclave before discarding.)
4. Treat the cell suspension with caution—these are viable organisms. Plate the suspension **immediately** on the recommended agar media.

## D. Fluorescence Equipment with Mercury Arc Lamps

1. Make certain that power packs for mercury arc lamps are electrically grounded.
2. Never open the lamp housing on mercury arc equipment while bulb is burning or hot.
3. Always have a glass filter in place before looking into the microscope.

4. If a mercury bulb explodes, quickly unplug the equipment and leave the room. Do not allow anyone to enter the room for a minimum of 1 h while the mercury vapors settle.

#### E. Personal Hygiene

1. A laboratory working with the LDB should be marked with a biohazard warning sign; access should be controlled by the laboratory supervisor.
2. Use mechanical pipetting devices for transferring liquid materials. **Mouth pipetting is prohibited.**
3. Examine glassware, and discard all pieces with rough or chipped edges that might puncture the skin.
4. Wear gloves if there is any broken skin (cut, scratch, etc.) on the hands. Wear a mask if there is any chance of producing an aerosol.
5. **Do not eat, drink, smoke, or store food in the laboratory.**
6. Always wash hands after handling specimens and cultures, and before leaving the laboratory area.

#### F. Medical Surveillance

Careful medical surveillance should be maintained for everyone working with the LDB or in the same general area.

1. Obtain and store in a serum bank control or baseline sera from all personnel.
2. Carefully monitor all personnel for febrile illness which might indicate a laboratory-associated LDB infection.

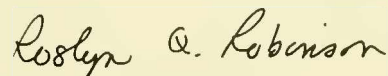
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## II. LABORATORY SERVICES

### A. Policy

*The laboratories of CDC serve as a national reference facility and accept specimens only from state public health laboratories and Federal facilities. Specimens which cannot be adequately examined locally should be sent to the appropriate state laboratory where they will either be processed or, at the discretion of the state laboratory director, forwarded to CDC. Acceptance of diagnostic specimens by CDC directly from private physicians or institutions and local health departments is authorized only under special circumstances including prior agreement between CDC and the state laboratory director.*



Roslyn Q. Robinson, Ph.D.  
Director, Bureau of Laboratories

### B. Reference Material

#### 1. Cultures

Cultures of Legionnaires' disease bacteria (LDB) can be obtained by laboratories for control purposes. However, before authorizing the distribution of a culture, CDC requires that the laboratory director submit a written request which affirms that the organism will be studied only under suitable containment conditions; that the cultures will be handled by qualified microbiologists exercising appropriate care; and that no subcultures of this organism will be distributed to other laboratories. These precautions are necessary to insure the safety of all concerned until the mode of spread, habitat, infectivity, and other characteristics of this organism are more clearly defined. The letter of request should be sent to:

Center for Disease Control  
Attention: Albert Balows, Ph.D.  
Director, Bacteriology Division  
Bureau of Laboratories  
Atlanta, Georgia 30333  
Telephone (404) 329-3711

When the requirements above are met, an LDB culture will be mailed. **Open and inspect the culture upon arrival.** Most of our reference cultures are lyophilized and sealed under vacuum, but some are currently mailed on agar slants. Stock culture storage on agar media is discussed in the chapter by Feeley et al., a blood-freeze method is detailed in the Appendix, and lyophilized cultures are discussed in the section above on Laboratory Safety.



## 2. Reagents

The Legionnaires' disease direct and indirect fluorescent antibody research reagents listed below are available from:

Center for Disease Control  
Attention: Biological Products Division  
Bureau of Laboratories  
Atlanta, Georgia 30333  
Telephone (404) 329-3355

Biological Products Requisition forms (see sample) should be used to order all such reagents. Supplies of forms can also be ordered from the above address.

At present there are no restrictions on distribution; however, when these reagents become commercially available, they will be distributed in accordance with the established reagent distribution policy stated in the next section.

Federal agencies are required to supply a purchase order with each requisition and should contact the above address for a price quotation.

Catalog Number	DIRECT FA PRODUCTS	Packing Unit
BE1578	Globulin FITC Polyvalent (Serogroups 1, 2, 3, 4)	2 ml
BE1538	Globulin FITC Serogroup 1, Knoxville Isolate	2 ml
BA1629	Antigen	1 ml
BE1514	Globulin FITC Serogroup 2, Togus 1 Isolate	2 ml
BA1630	Antigen	1 ml
BE1575	Globulin FITC Serogroup 3, Bloomington Isolate	2 ml
BA1631	Antigen	1 ml
BE1576	Globulin FITC Serogroup 4, Los Angeles 1 Isolate	2 ml
BA1632	Antigen	1 ml
BE1567	Globulin FITC Normal	1 ml
INDIRECT FA PRODUCTS		
KK0003	Reagent Set	
	Antigen Serogroup 1, Philadelphia 1 Isolate	2 ml
	Diluent Normal Yolk Sac 3%	45 ml
	Globulin FITC Labeled Anti-Human	4 ml
	Serum Serogroup 1 Positive Control	1 ml
BA1533	Antigen Serogroup 1, Philadelphia 1 Isolate	2 ml
BA1515	Antigen Serogroup 2, Togus 1 Isolate	2 ml
BS1525	Serum Serogroup 2, Positive Control	1 ml
BG1566	Diluent Normal Yolk Sac 3%	45 ml
BE1537	Globulin FITC Labeled Anti-Human	2 ml

# BIOLOGICAL PRODUCTS REQUISITION

SAMPLE

Ordering Agency Complete Bold-lined Section ONLY

REQUESTED BY <b>J.R. Smith</b>	APPROVED BY <b>A.R. Jones</b>	DATE <b>9/28/78</b>	IF APPLICABLE PHS GRANT NO.		COC ADDRESS <b>CENTER FOR DISEASE CONTROL ATTN: BIOLOGICAL PRODUCTS DIVISION ATLANTA, GEORGIA 30333</b>	
(NAME & ADDRESS OF CONSIGNEE)  <b>Dr. Adam Smith Microbiology Laboratory State of Maine Health Department Portland, Maine 78102</b>			FED P.O. NO.		CONSIGNEE CUSTOMER NO.	REIMB REQUESTOR CUSTOMER NO.
			COC 'CAN' NO.		TYPE OF SHIPMENT <input type="checkbox"/> (RN) NO CHARGE <input type="checkbox"/> (RR) REIMB & CONSIGNEE <input type="checkbox"/> (RC) REIMB REQUESTOR & NOT CONSIGNEE <input type="checkbox"/> (RM) REMOVE FORM INVENTORY	
			REIMBURSABLE REQUESTOR (NOT CONSIGNEE)			
TELEPHONE NO. <b>404-292-3368</b>			REPLACEMENT FOR PREVIOUS SHIPMENT <input type="checkbox"/> (Y) YES <input type="checkbox"/> (N) NO CREDIT <input type="checkbox"/> (W) WHO <input type="checkbox"/> (P) PAHO <input type="checkbox"/> (O) OTHER REIMB: <input type="checkbox"/> (P) P.O., COC, WHO <input type="checkbox"/> (G) GRANT			
			DATE RECEIVED	INVOICE DATE	DATE SHIPPED	
			INVOICE NO. TRAILER			
PRODUCT NAME		QUANTITY ORDERED	CATALOG NO.	LOT NO.	LOCATION	QUANTITY SHIPPED
1. LD IFA Kit		1 Set	KKG003			
2. LD Anti-human Globulin		1 Vial	BE 1537			
3.						
4.						
5.						
6.						
7.						
8.						
9.						
10.						
11.						
12.						

COMMENTS

a. **Policies Governing the Distribution of CDC Products**

The types of products produced and distributed by the Biological Products Division, Bureau of Laboratories, CDC, are described below. Also included are a listing and brief description of authorized recipients. Further inquiries should be sent to:

Center for Disease Control  
Attention: Biological Products Division  
Bureau of Laboratories  
Atlanta, Georgia 30333  
Telephone (404) 329-3355

(1) **Products**

(a) **In Vitro Diagnostic Reference**

Reference products are provided to consumers so that they can determine whether similar products prepared by commercial manufacturers or in noncommercial laboratories meet CDC specifications. For this purpose, only small volumes, usually one packing unit, are distributed.

(b) **In Vitro Diagnostic**

When there is a public health need for certain diagnostic products which can neither be purchased nor prepared in a noncommercial laboratory, CDC may be able to supply quantities sufficient for diagnostic use.

(c) **Research and Investigational**

When reagents are distributed for research and other investigations, the requester agrees to provide, upon request, information about the specific use of products and reports of the results obtained. Some of these products may not meet the requirements of published CDC Specifications.

(2) **Authorized Recipients**

The following are authorized to receive products:

1. **OTHER FEDERAL AGENCIES.** These agencies receive products on a reimbursable basis.
2. **STATE, TERRITORIAL, AND LOCAL PUBLIC HEALTH AGENCIES.**
3. **INTERNATIONAL PUBLIC HEALTH AGENCIES,** such as the World Health Organization (WHO) and the Pan American Health Organization (PAHO).
4. **PUBLIC HEALTH SERVICE GRANTEES.** Grantees will be provided only those products needed for studies covered by their grants. They are not eligible to receive other products.



5. **COMMERCIAL PRODUCERS OF IN VITRO DIAGNOSTIC PRODUCTS.** Manufacturers will be provided with CDC reference products for use in evaluating lots of commercial products to determine whether they meet the CDC Specifications.

6. **COLLABORATING RESEARCHERS.** This group includes private investigators and investigators in universities, medical centers, and other teaching and research facilities who are studying problems of interest to CDC.

### 3. Tissues

Control tissue for the modified Dieterle silver impregnation stain is available.

Sufficient lung tissue containing the Legionnaires' disease bacterium is not available for distribution. The control tissue distributed for this procedure contains a spirochete. This material consists of a paraffin-embedded block and a matching Dieterle-stained slide. A reference slide of Dieterle-stained lung tissue from a known case of Legionnaires' disease will also be sent on request. Requests should be sent to:

Center for Disease Control  
Attention: Mrs. Pat Greer  
Control Tissue Repository  
Pathology Division  
Bureau of Laboratories  
Atlanta, Georgia 30333  
Telephone (404) 329-3623

### 4. Chemicals

Ferric pyrophosphate for Feeley-Gorman (F-G) and charcoal yeast extract (CYE) agar media is available on request from:

Center for Disease Control  
Attention: Biological Products Division  
Bureau of Laboratories  
Atlanta, Georgia 30333  
Telephone (404) 329-3353

## C. Reference Diagnostic Services

### 1. Indirect Immunofluorescence (IF) Test

As a general rule, only paired (or serial) acute-phase and convalescent-phase sera should be submitted. Ideally, the acute-phase serum should be collected during the first week of illness, and the convalescent-phase serum should be obtained 3 wk after onset of illness. Titers usually peak at approximately 5 wk after onset.

Place the acute-phase serum in a 1-dram screw-capped glass vial. Seal the vial with waterproof tape, label for proper identification, and store at 4°C. Prepare a second vial for the convalescent-phase serum. When both sera are ready, tape the two vials together to avoid separation in transit and mail them with CDC Form 3.203 to CDC. If you subsequently receive additional convalescent-phase sera from the same patient, please submit them to CDC with appropriate identification, a CDC Form 3.203, and a statement that previous sera from this patient were submitted for the indirect IF test.

If we do not receive an acute-phase serum collected during the first week of illness, we will perform the indirect IF test on a single serum collected at least 14 days after onset of illness. However, because a single serum specimen obtained before this time and submitted for indirect IF testing will not be evaluated without justification, we strongly urge that every attempt be made to obtain a second sample. Sera from blood samples obtained just before a patient dies or during autopsy will be tested, with the understanding that we may not find a significant titer.

Address requests for indirect IF and other serologic tests to:

Center for Disease Control  
Attention: Dr. Hazel W. Wilkinson  
Special Immunology Laboratory  
DASH—Unit 10  
Bacterial Immunology Branch  
Bureau of Laboratories  
Atlanta, Georgia 30333  
Telephone (404) 329-3929

## 2. Isolation from Clinical Specimens

Portions of lung specimens submitted for isolation of possible LDB should be carefully selected. To the extent possible, pathologists should select areas of dense consolidation or areas with caseating necrosis, because such lung tissue is most likely to contain LDB. Specimens should be packaged and mailed according to the Interstate Quarantine Regulation (42 CFR Part 72) for shipping etiologic agents and should be surrounded by enough cold-pack artificial ice to keep the material cold until it arrives at CDC. Shipping specimens frozen or in dry ice is not necessary. Tissue fixed in Formalin or other fixatives is obviously unsuitable for isolation attempts but will be examined with direct immunofluorescence.

Sputa or other respiratory tract fluid specimens, **except** pleural fluids, cannot be accepted for isolation attempts unless authorized in advance.

Address requests and specimens to:

Center for Disease Control  
Attention: Dr. Roger McKinney  
Immunofluorescence Section  
DASH—Unit 11  
Analytical Bacteriology Branch  
Bureau of Laboratories  
Atlanta, Georgia 30333  
Telephone (404) 329-3563

## 3. Direct Immunofluorescence (IF) Test

In addition to the two reference diagnostic services described above, the Bacteriology Division provides reference diagnosis and consultation on the use of specific immunofluorescent conjugates for the direct staining of LDB in fresh or Formalin-fixed tissues and other clinical specimens. Neutral Formalin is the best fixative to use if the tissue is to be examined with direct IF. Such tissues do not require refrigeration and should not be frozen because they are then unsuitable for pathology studies.

Address requests and specimens to:

Center for Disease Control  
Attention: Dr. Roger McKinney  
Immunofluorescence Section  
DASH—Unit 11  
Analytical Bacteriology Branch  
Bureau of Laboratories  
Atlanta, Georgia 30333  
Telephone (404) 329-3563



#### **4. Identification of Culture Isolates**

Cultures suspected of being the LDB will be confirmed or identified.

Address requests and specimens to:

Center for Disease Control  
Attention: Dr. Robert E. Weaver  
Special Bacteriology Section  
DASH—Unit 17  
Clinical Bacteriology Branch  
Bureau of Laboratories  
Atlanta, Georgia 30333  
Telephone (404) 329-3248

#### **5. Histopathological Examination**

Formalin-fixed tissues from patients with suspected LD will be examined. Either wet or paraffin-embedded tissues are acceptable.

Address requests and specimens to:

Center for Disease Control  
Attention: Dr. Martin D. Hicklin  
Pathology Division  
DASH—Unit 09  
Bureau of Laboratories  
Atlanta, Georgia 30333  
Telephone (404) 329-3136

### **D. Submitting Reference Specimens**

#### **1. Information Required**

Specimens submitted to CDC for any or all of the above-listed reference diagnostic services must be accompanied by the following patient information on CDC Form 3.203 (see below):

- (a) Patient's name
- (b) Patient's birth date
- (c) Patient's sex
- (d) Referring physician's clinical diagnosis
- (e) Date of onset of illness
- (f) Dates sera were drawn
- (g) Whether illness was fatal
- (h) Highest temperature recorded for patient
- (i) Information on other specimens from the patient which have been submitted separately to a state health department laboratory or to CDC

#### **2. Transporting**

The reliability of laboratory test results largely depends upon the care and thought used in the collection, handling, and shipment of diagnostic specimens.

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE  
PUBLIC HEALTH SERVICE  
CENTER FOR DISEASE CONTROL  
Bureau of Laboratories  
Atlanta, Georgia 30333

STATE HEALTH DEPARTMENT LABORATORY ADDRESS

STATE HEALTH  
DEPT. NO.:

DATE SENT TO Month Day Year  
CDC:

NAME AND ADDRESS OF PHYSICIAN OR ORGANIZATION:

PATIENT IDENTIFICATION

Hospital No.

NAME: Last (18-37) First (38-47) Middle Initial (48)

BIRTHDATE: (49-54)  
Month Day Year

SEX: (55)

CLINICAL DIAGNOSIS: (56-57)

ASSOCIATED ILLNESS: (58-59)

DATE OF ONSET (Mo. Da. Yr.) (60-65)

FATAL? (66)  
☐ YES ☐ NO

(Below this line for CDC Use Only)

CDC NUMBER

DATE RECEIVED (12-17)

UNIT FY (3-4) NUMBER (5-10) SUF (11)

Month Day Year

REVERSE SIDE OF THIS FORM MUST BE COMPLETED

Type Specimen

THIS FORM MUST BE EITHER PRINTED OR TYPED

PLEASE PREPARE A SEPARATE FORM FOR EACH SPECIMEN

(Consider paired sera one specimen)

D.A.S.H.

HSM 3.203-R  
6-72

Date Reported

Mo. Day Yr.

0 3  
(12-13)

(14-19)

Comments:

(40-41)

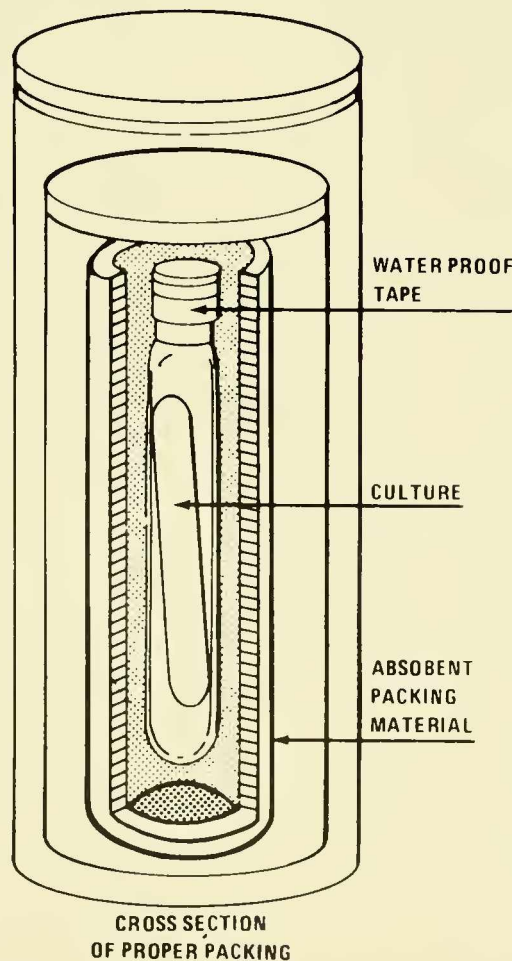
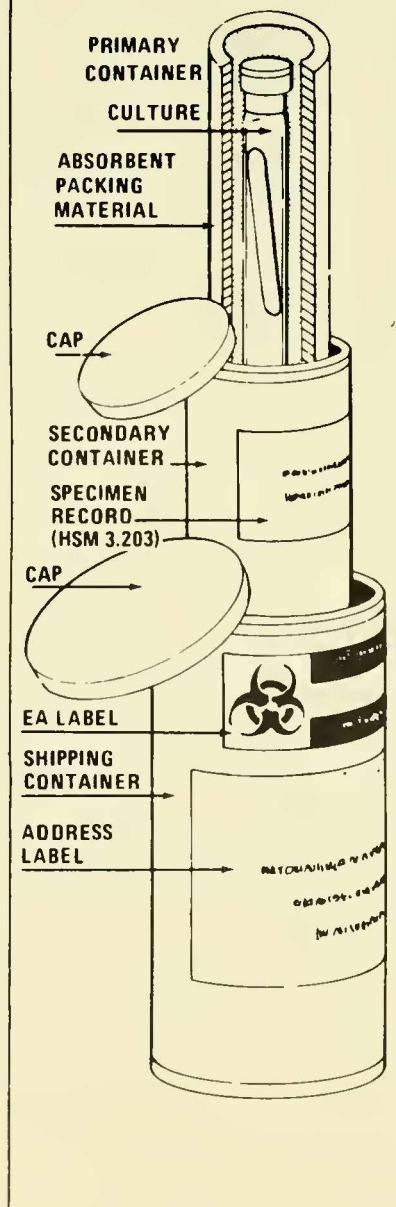
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PLEASE DO NOT USE BLUE INK





# PACKAGING AND LABELING OF ETIOLOGIC AGENTS



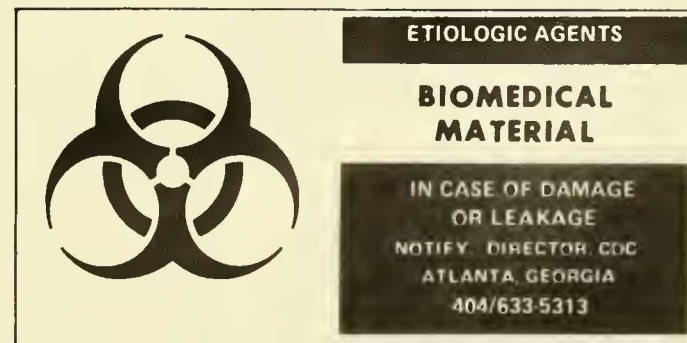
The Interstate Quarantine Regulations (Code of Federal Regulations, Title 42, Part 72.25, Etiologic Agents) was revised July 30, 1972, to provide for packaging and labeling requirements for etiologic agents and certain other materials shipped in interstate traffic.

The figure diagrams packaging and labeling of etiologic agents in volumes less than 50 ml in accordance with the provisions of subparagraph (C) (1) of the cited regulation.

The Etiologic Agents—Biomedical Material label (see subparagraph (C) (4) of regulations) which must be placed on all shipments of etiologic agents is depicted below. The label must be 2 inches high by 4 inches long and printed in red ink on white stock. For further information on any provision of this regulation contact:

Center for Disease Control  
Attn: Office of Biosafety  
1600 Clifton Road  
Atlanta, Georgia 30333

Telephone: (404) 329-3311



The following are practical procedures which the CDC laboratory staff have found insure viability of the infectious agent and provide maximum protection for those handling the shipment, both in transit and after it arrives at the laboratory. These procedures should be followed when specimens are shipped to CDC for testing.

a. **Packing**

Pack specimens so that they are protected in transit and so that the personnel who handle the packages are also protected.

(1) Enclose the specimen in a 2-ml or larger screw-capped tube or vial. (This rule does not apply to bacterial cultures submitted for identification; they should be submitted as slant or stab cultures.) Seal the tube or vial with waterproof tape.

*NEVER mail clinical specimens or cultures in petri plates!*

*NEVER enclose dry ice in hermetically sealed containers!*

*DO NOT use plastic vials for routine shipment of specimens!*

(2) Place the tube in a watertight shipping can. Pack absorbent cotton or other suitable absorbent material around the tube to minimize shock and contain any leakage. Do not use particulate material for this purpose. If several tubes are placed in the same can, cushion them by wrapping each individually in paper towels or cotton. Never wrap CDC Form 3.203 around the tube containing the specimen; wrap it around the secondary container (see Figure).

(3) In an outer cardboard shipping container, pack the secondary container surrounded with crumpled newspaper or other shock-resisting insulating material. CDC strongly recommends using a special commercially available styrofoam-lined cardboard shipping container instead of ordinary cardboard containers. Seal the outer shipping container securely and affix a properly completed address label.

b. **Shipping**

(1) When CO<sub>2</sub> is used as a refrigerant, mark the outer shipping container in accordance with applicable Department of Transportation regulations and Air Transport Association tariffs.

(2) Affix the Etiologic Agent/Biological Materials label to an exterior surface of any container for etiologic agents (cultures, virus suspensions, bacterial toxins) in accordance with Title 42, Code of Federal Regulations, Section 72.25.

(3) If you are shipping specimens for long distances, send them by an expedited package service to assure prompt arrival. (Avoid air freight because it is often delayed.)

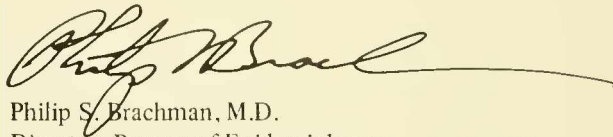
(4) With each specimen, submit a completed CDC Form 3.203. Furnish as much of the information requested on the form as you possibly can, because it facilitates faster and more reliable diagnostic service.

(5) When possible, ship specimens so that they will arrive at the laboratory at the beginning or middle of the workweek and **not** just before or during a weekend or holiday.

### III. EPIDEMIOLOGIC SERVICES

#### A. Policy

*The Center for Disease Control provides epidemiologic assistance to state health departments at their request and has responsibility for interstate disease outbreaks. Consultation is freely provided to individual members of the health professions and lay public on an ad hoc basis, although formal assistance is coordinated through state health departments.*



Philip S. Brachman, M.D.  
Director, Bureau of Epidemiology

#### B. Epidemiologic Consultation and Assistance

Legionnaires' disease may occur as sporadic cases or in outbreaks. Because some outbreaks of Legionnaires' disease may be terminated by identifying and eliminating a common source of infection (e.g., a contaminated air-conditioning cooling tower or evaporative condenser), consultation may be desired concerning a cluster of cases of Legionnaires' disease. This can be obtained directly from the state epidemiologist, whose name and telephone number can be obtained from the department of health or comparable agency of the state government, or from:

Center for Disease Control  
Attention: Special Pathogens Branch  
Bacterial Diseases Division  
Bureau of Epidemiology  
Atlanta, Georgia 30333  
Telephone: (404) 329-3687

The size of a cluster necessitating consultation will vary greatly depending on how tightly the cases are clustered in time and space. Occasionally examination of tight clusters as small as two to four cases may provide information of public health importance.

#### C. Clinical Consultation

Specific antibiotic therapy may decrease the case-fatality ratio associated with Legionnaires' disease, so early clinical and laboratory diagnosis is of great importance. Consultation on the clinical aspects of suspected cases of Legionnaires' disease is available from the state epidemiologist, whose name and telephone number can be obtained from the department of health or comparable agency of the state government, or from:

Center for Disease Control  
Attention: Special Pathogens Branch  
Bacterial Diseases Division  
Bureau of Epidemiology  
Atlanta, Georgia 30333  
Telephone: (404) 329-3687





# **PART FIVE**

## **Appendix**





## Appendix

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## I. CHROMOGENIC CEPHALOSPORIN TEST FOR $\beta$ -LACTAMASE PRODUCTION

Results of degradation studies and the chromogenic cephalosporin test have documented that the Legionnaires' disease bacterium produces a  $\beta$ -lactamase. The enzyme is more active on cephalosporin than on penicillin, and is not at all active on cefoxitin or cefuroxime (newer cephalosporins not yet approved in the United States but used in other countries).

The procedure for performing the chromogenic cephalosporin test for  $\beta$ -lactamase production follows.

### A. Reagents

#### 1. Phosphate Buffer, pH 7.0

- a. M/15  $\text{KH}_2\text{PO}_4$  . . . . . 9.07 g/liter distilled water
- b. M/15  $\text{Na}_2\text{HPO}_4$  . . . . . 9.46 g/liter distilled water
- c. Mix 39.2 ml of the potassium phosphate solution with 60.8 ml of the sodium phosphate solution. This buffer is stable for several weeks at room temperature.

#### 2. Cephalosporin Substrate

- a. Cephalosporin 87/312 is available only from:  
Glaxo, Ltd.  
Greenford, Middlesex UB6 OHE  
England
- b. Dissolve 10 mg of cephalosporin 87/312 in 1 ml of dimethylsulfoxide (DMSO). Dilute with phosphate buffer, pH 7.0, to a concentration of 500  $\mu\text{g/ml}$ . The substrate is yellow when viewed in a microdilution plate, but larger volumes may appear more orange. However, the red reaction which constitutes a positive test result is easily discerned. This substrate is stable at 4°-10°C for many weeks.

### B. Procedure

#### 1. In a Microdilution Plate or Small Tube:

- a. Place 0.05 ml of cephalosporin substrate in a well of a microdilution plate (or in a small tube).
- b. Use a loop to remove several colonies of the test organism from the agar surface. Make a turbid cell suspension in the cephalosporin substrate.
- c. Mix the substrate and cells for 1 min. Observe for color change immediately, after 10 min, and after 1 h.
- d. If the test organism produces  $\beta$ -lactamase, the color of the substrate will change from yellow to red.  $\beta$ -lactamase-producing *Haemophilus influenzae* or *Neisseria gonorrhoeae* isolates usually turn the substrate red in less than 10 min, but staphylococci may take an hour.
- e. Run the test with a known  $\beta$ -lactamase-producing strain and a non  $\beta$ -lactamase-producing strain of *N. gonorrhoeae*, *H. influenzae*, or *Staphylococcus aureus*.

## 2. On an Agar Plate:

- a. The agar plate should contain the test organism in pure culture.
- b. Place a drop (approximately 0.05 ml) of the cephalosporin substrate on an area of bacterial growth, and tilt the plate slightly so that the drop drains across the surface of the growth.
- c. If the test organism produces  $\beta$ -lactamase, the substrate along the path will turn red; if it is  $\beta$ -lactamase negative, the substrate along the path will be yellow.
- d. Although the red reaction on the plate is not as easily seen as that in the microdilution well, it is generally quite obvious. If the analyst is unsure about the reaction, the test should be repeated in a microdilution well or small tube.
- e. Usually a positive reaction can be read immediately, but the plate should be reexamined after 10 min.

## C. Precautions

1. Primary isolation media (e.g., modified Thayer-Martin medium) may grow  $\beta$ -lactamase-producing bacteria in addition to the organism of interest (e.g., Legionnaires' disease bacterium), which may lead to false-positive results.
2. Clinical specimens (e.g., pleural fluids) cannot be evaluated directly with the chromogenic cephalosporin test.



## II. CULTIVATION OF THE LEGIONNAIRES' DISEASE BACTERIUM IN YOLK SAC OF EMBRYONATED HENS' EGGS

**Age of Eggs:** 6-7 Days

**Type of Eggs:** Antibiotic-free

**Inoculation:** Equipment - 1½" Needle  
Volume - 0.5 ml  
Diluent - Phosphate buffered  
saline (PBS), pH 7.2

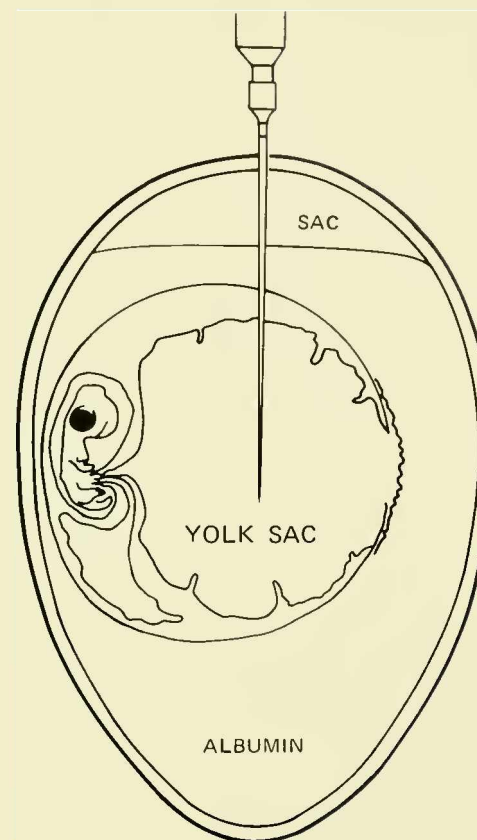
**Incubation:** Temperature - 35°C  
Length - Varies 4-10 days  
Observations - Note deaths: Discard those  
dying up through 3rd day. Harvest  
those dying from 4th through 10th days.

**Harvest:** Remove shell and pour out contents.  
Detach yolk sac from embryo. Aseptically strip  
membrane to remove yolk. Make slide smear  
of yolk sac.

**Evaluation:** Observe agent in Giménez-stained smears.  
Bacterial cultures. Thioglycollate broth  
Trypticase soy  
Blood agar  
Feeley-Gorman agar  
Charcoal yeast extract agar

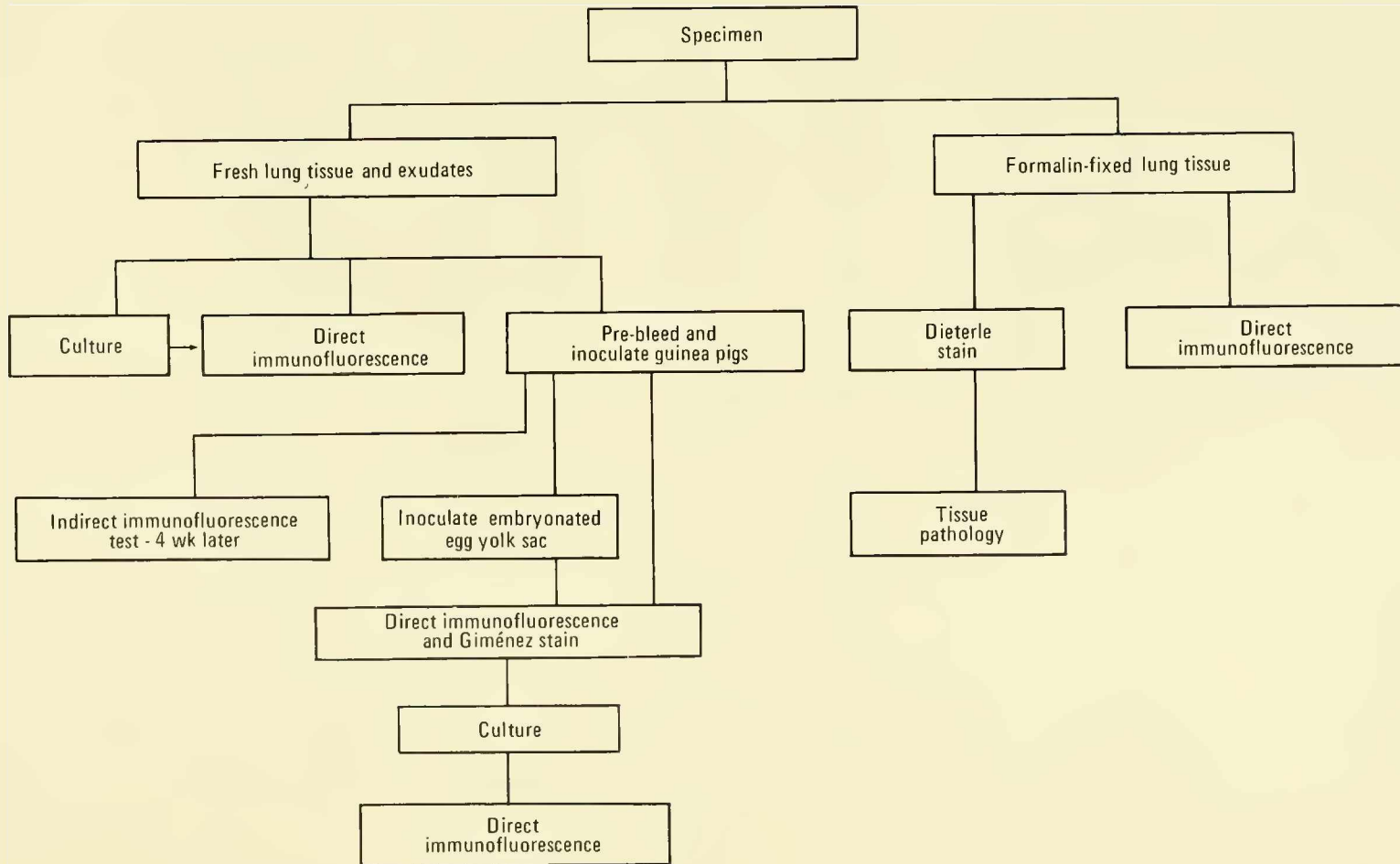
Serology - Direct or indirect immunofluorescence  
test with standard antisera

Transmissible by serial passage



Yolk Sac Inoculation  
Six-Day-Old Chick Embryo

### III. Detection of the Legionnaires' Disease Bacterium in Clinical Specimens



#### IV. BLOOD-FREEZE STORAGE OF BACTERIA

Some stock cultures should be maintained for checking each new lot of media and conjugate and for staining along with each batch of unknowns.

These stock cultures can be obtained from positive clinical specimens or from a reference laboratory. The stock cultures can be stored as described by Feeley et al. in this manual or by the blood-freeze method described below.

##### A. Supplies

###### 1. Freeze tubes

The freezing tubes should be made of Pyrex glass (e.g., Corning #9820, size 6 X 50 mm). The Pyrex will stand the sudden change in temperature to which it will be subjected. New tubes should be boiled in three changes of distilled water to remove any deleterious substance. These tubes are shaken thoroughly to remove the water, dried, plugged, and sterilized in a hot air oven. The cultures can be identified by using ¼- X 1-in strips of waterproof adhesive tape on which the name or number of the culture has been typed or printed in India ink.

###### 2. Storage boxes

Arrange for some type of storage boxes that will fit into the available freezer space and in which the cultures can be systematically filed. Storage boxes can be constructed of 3/8-in marine waterproof plywood, plastic, or cardboard.

##### B. Procedure

Transfer the stock cultures to charcoal yeast extract agar slants, and incubate them at 35°C for 48-72 h, or to Feeley-Gorman agar slants, and incubate for 3-5 days at 35°C in air plus 2.5% CO<sub>2</sub>.

###### 1. Freezing

After growth is obtained, add 1 to 2 ml of sterile defibrinated rabbit or sheep blood aseptically to each slant. Use capillary pipettes to suspend the growth in the blood, then aspirate and deliver approximately 0.2 ml to each of the previously numbered freezing tubes. If cotton plugged tubes are used, cut off excess cotton and flame the lip. Transfer the tubes to their respective numerical slots in the storage box. Store at -50°C.

###### 2. Recultivation

Do not thaw and refreeze frozen suspensions. To recover the organisms, remove one tube from the freezer, and thaw the contents rapidly by holding the tube tightly in the palm of the hand or by placing the tube in a 35°C water bath. Draw off the thawed suspension with a capillary pipette and transfer to a suitable culture medium.



## V. IMMUNOFLUORESCENCE BUFFERS AND MOUNTING FLUIDS

Some immunofluorescence techniques utilize phosphate buffers and others call for carbonate buffers. Both systems are recommended in this manual and presented below.

### A. Phosphate Reagents

#### 1. Phosphate Buffered Saline (PBS), pH 7.6, 0.01 M:

- a. Concentrated Stock Solution (pH will not be 7.6):

Na<sub>2</sub>HPO<sub>4</sub> (anhydrous; reagent grade) . . . . . 12.36 gm  
 NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O (reagent grade) . . . . . 1.80 gm  
 NaCl (reagent grade) . . . . . 85.00 gm  
 Distilled water to make final volume . . . . . 1 liter  
 Dibasic salt dissolves much more readily in water at 37°C  
 than at 25°C.

- b. Working Solution (pH 7.6; 0.01 M buffer; 0.85% NaCl):

Concentrated stock solution . . . . . 100 ml  
 Distilled water to make final volume . . . . . 1 liter

#### 2. Phosphate Buffered Saline (PBS), pH 7.2:

0.15 M KH<sub>2</sub>PO<sub>4</sub> . . . . . 24 ml  
 0.15 M Na<sub>2</sub>HPO<sub>4</sub> . . . . . 76 ml  
 0.85% NaCl . . . . . 100 ml

#### 3. Buffered Glycerol Mounting Fluid, approx. pH 9.0:

Glycerol, reagent grade . . . . . 90 ml  
 0.2 M Na<sub>2</sub>HPO<sub>4</sub> . . . . . 10 ml

### B. Carbonate Reagents

#### 1. Carbonate-Bicarbonate Buffer, pH 9.0, 0.5 M:

- a. 0.5 M Na<sub>2</sub>CO<sub>3</sub> . . . . . 5.3 g/100 ml of distilled water  
 b. 0.5 M NaHCO<sub>3</sub> . . . . . 4.2 g/100 ml of distilled water  
 c. Mix 4.4 ml of solution (a) with the 100 ml of solution (b). Theoretically, a pH of 9.0 should result from this mixture; however, as much as 17.0 ml of solution (a) may have to be added to the 100 ml of solution (b).

#### 2. Glycerol Mounting Fluid:

- a. 0.5 M Carbonate-bicarbonate buffer, pH 9.0 . . . . . 1 part  
 b. Neutral glycerol, reagent grade . . . . . 9 parts  
 c. Combine and mix by stirring (do not shake).

## VI. NORMAL CHICKEN YOLK SAC (NYS)

A normal chicken egg yolk sac suspension is used in the indirect immunofluorescence test for antibody to the Legionnaires' disease bacterium (see chapter by Wilkinson et al.). The suspension is needed for the preparation of working dilutions of the bacterial antigens and for the initial dilution in titration of sera.

1. Harvest the yolk sac from 12- to 14-day-old embryonated hens' eggs. Separate as much of the yolk material from the sac as possible. The average yield of yolk sac per egg is 4 g.
2. Place the yolk sacs in an equal volume of 0.02 M phosphate buffered saline, pH 7.2 (PBS), containing 0.05% sodium azide.
3. Homogenize in an Ominimixer or Waring blender, and filter through two layers of gauze.
4. Dilute the suspension with PBS containing 0.05% sodium azide to 0.5% (w/v) for antigen preparation or 3% for use as a diluent in the FA test.

## VII. McFARLAND NEPHELOMETER DENSITY STANDARDS

Add 1% barium chloride and 1% sulfuric solutions to 10 uniform tubes in the proportions shown below. Seal to prevent any evaporation, and label the tubes. Before using standards, shake tubes well to resuspend the precipitate.

McFarland standard #	1% BaCl <sub>2</sub> solution (ml)	1% H <sub>2</sub> SO <sub>4</sub> solution (ml)	Appx. density of bacteria rep. (million/ml)	International Units (I.U.) of Opacity
1	0.1	9.9	300	3
2	0.2	9.8	600	7
3	0.3	9.7	900	10
4	0.4	9.6	1200	12
5	0.5	9.5	1500	15
6	0.6	9.4	1800	—
7	0.7	9.3	2100	20
8	0.8	9.2	2400	—
9	0.9	9.1	2700	—
10	1.0	9.0	3000	30

# **PART SIX**

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